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Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance

PRINCIPAL INVESTIGATOR:
Dr. Thomas Neylan

CONTRACTING ORGANIZATION:
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San Francisco, CA 94109

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Thomas Neylan, M.D.

email: thomas.neylan@va.gov

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14. ABSTRACT

In Year 5, we completed study enrollment. In total, 203 participants received the study intervention. Safety reports were sent to the study drug sponsor Actelion on a monthly basis. Both blinded and unblinded monitoring visits of study procedures and facilities are ongoing and should be completed by the end of October 2014.We are currently working on data cleaning, quality control efforts, and edit checks and hope to begin analyses in November 2014.

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ANNUAL PROGRESS REPORT
September 30, 2014
Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance
USAMRMC Grant W81XWH-09-2-0080
Thomas Neylan, M.D., Principal Investigator

INTRODUCTION

An integrated translational study will be conducted to examine the effect of a novel hypocretin/orexin antagonist, almorexant (ALM), compared to a standard hypnotic, zolpidem (ZOL), and placebo (PBO) on neurocognitive performance at peak concentration post dosing. The human study component (Task 1; responsible individual: Thomas Neylan, M.D.) will establish whether ALM is superior to ZOL in relation to neurocognitive side effects. It is hypothesized that healthy human subjects receiving 10mg of zolpidem will show greater impairment in neurocognitive performance compared to subjects receiving 100mg or 200mg doses of almorexant or placebo. Study subjects (n=200) will receive a randomly assigned, one-time dose of the study drug in an inpatient hospital setting. A battery of neurocognitive, objective alertness, and subjective symptom assessments will be administered prior to and following dosing. Assessments to be administered were selected based upon their demonstrated sensitivity to sleep-inducing agents and their military relevance. The animal study component (Tasks 2 – 5; responsible individual: Thomas Kilduff, Ph.D.) will compare the neural circuitry that underlies the activity of the abovementioned compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

BODY

Progress associated with each task outlined in the approved Statement of Work is listed below:

Task 1: *Test the hypothesis that healthy human subjects receiving ZOL 10mg will show greater impairment in neurocognitive performance compared to subjects receiving PBO or the 2 doses (100mg, 200mg) of ALM.*

The Task 1 subtasks listed below have been completed prior to or during Year 5:

Subtask #1: Write Protocol

The study protocol was finalized during Year 1, and modifications were made to the protocol during Year 2, Year 3, Year 4 and Year 5. The current version of the protocol is included in Appendix 1.

Subtask #2: Obtain Scientific and Human Use Approvals

Study documentation was submitted to the appropriate Institutional Review Boards (IRBs) and the Food and Drug Administration (FDA) for approval prior to the end of Year 1. All human subjects approvals were obtained during Year 2. Approval timelines are detailed below:

- IRB Approval:
  Initial Approval

The University of California, San Francisco Committee on Human Research (UCSF CHR) provided initial approval on October 29, 2010. The Department of Veterans Affairs Medical Center Research and Development Committee (VA R&D Committee) provided approval on January 6, 2011. The U.S. Army Medical Research and Materiel Command Human Research Protection Office (USAMRMC HRPO) provided initial approval on March 9, 2011.

Approval of Amendments

An amended Investigator's Brochure was provided by Actelion Pharmaceuticals on March 23, 2011 which necessitated revisions to the study protocol and informed consent document. Enrollment could not begin until all Institutional Review Boards approved the revised study documents. The UCSF CHR and the VA R&D Committee approved the revisions on May 3, 2011. The USAMRMC HRPO approved the revisions on May 10, 2011, at which point enrollment could be initiated. Informed consent and VA HIPAA documents were revised and approved by UCSF CHR on February 7, 2012 (modifications included giving participants the option to consent to be contacted for participation in other research studies within the Stress & Health Research Program, as well as requesting that participants continue filling out a sleep diary and wearing an actigraph during the hospital portion of the study). The informed consent was again revised and approved by UCSF CHR on October 9th, 2013 and on January 15th, 2013 (modifications included providing participants with additional information related to FDA changes to the recommended dose of Zolpidem for women, allowing female participants to be dosed during either the follicular or luteal phase of menstruation, adding a VA consent form for use of picture and/or voice, compensation allocation changes, and updates to recruitment text). The informed consent was revised and approved by UCSF CHR on January 20, 2014. Modifications included the addition of an optional sleep monitoring device.

Continuing Review

An annual continuing review application was approved by the UCSF CHR on August 19, 2014, extending the study's approval expiration to September 2, 2015. Continuing review approval from the VA R&D Committee was received on August 28, 2014. All continuing review approvals were last submitted to a continuing review analyst at the USAMRMC HRPO on September 27, 2012 and will be submitted again as requested.

- Investigational New Drug Application (IND): At the conclusion of Year 1, an IND application was filed with the FDA in order to obtain approval to receive study drug from Actelion Pharmaceuticals. The IND went into effect on October 21, 2010. The study protocol was re-submitted to the FDA on May 18, 2011 following revisions in response to the Investigator's Brochure Amendment received from Actelion Pharmaceuticals in March, 2011. In accordance with FDA requirements, an annual progress report was submitted on January 13th, 2014 and the next report will be submitted by no later than December 20th, 2014.

Subtask #3: Purchase Study Related Equipment/Supplies

The majority of study related equipment (including sleep equipment, actigraphs, psychomotor vigilance tests, and neuropsychological testing supplies) was purchased and tested during Year 1. Further testing and piloting of the equipment was performed during the early part of Year 2. All remaining study supplies (including drug testing kits and additional sleep equipment) were purchased and tested during Year 2.

The study drug (provided by Actelion Pharmaceuticals) arrived onsite at the UCSF Medical Center pharmacy in March, 2011. An external unblinded monitor has been appointed to perform regular drug accountability checks to confirm that drug is stored properly and in accordance with expiration dates. The most recent pharmacy monitoring visits took place on April 3, 2014 and August 4, 2014. Clinical Trial Management Software was purchased during Year 3, and we began using this software in September of 2013 to manage recruitment. Likewise, PRANA sleep software was purchased in 2013.

Subtask #4: Train Laboratory Personnel

Key study personnel were hired and trained during Year 1, Year 2, Year 3 and Year 4.

Subtask #5: Collect Data on 200 Volunteers

Recruitment and enrollment efforts were initiated in May 2011 (Y2, Q3), following receipt of all regulatory approvals. We completed enrollment in Y5, Q4. Enrollment details are outlined below:

A.) Enrollment Progress During Year 5

Summary of Y5 Enrollment Progress	
Total Number of Responses to Advertising in Y5	598
Total Number of Participants who Completed Phone Screening Procedures in Y5	389
Total Number of Participants who Consented in Y5	126
Total Number Deemed Eligible in Y5	86
Total Number Enrolled in study and dosed with Study Medication in Y5	85

Table 1. Summary of Year 5 Enrollment Progress

- **Advertising**: Advertising efforts involved monthly postings on the internet and recruitment at nearby universities, with a particular focus on students in healthcare professions. These ads, particularly at local universities, have generated a substantial response rate, as approximately 598 individuals have shown an interest in the study throughout the past year. Throughout Year 5, 389 interested participants were screened by phone prior to being scheduled for full eligibility assessments. While these statistics mark a decrease from last year, we attribute this to the fact that a) we ended recruitment in the middle of Y5, Q4 and b) our study recruiter who joined the team at the end of Y4 was very efficient and brought in a higher ratio of screened to eligible participants.
- **Screening**: From 9/1/2013 to 8/31/14, 126 participants met phone screen requirements, provided informed consent, and were invited to take part in full screening procedures at

the San Francisco VA Medical Center. Screening procedures include a mental health screening, self-report questionnaires related to caffeine use, tobacco use, alcohol use, and sleep habits, a physical exam, urine drug and pregnancy screens, and a blood draw for hematology and serum chemistry panels.

- **Eligible Participants**: From 9/1/2013 to 8/31/14, 86 participants were identified as eligible. 85 completed their inpatient hospital stay and dosing procedures. As of the end of Y5, a total of 203 participants have enrolled in the study and have been dosed with the study drug.

B.) Problem Areas

- **Miscellaneous Challenges**

  We experienced a number of unexpected delays in Y5. In early February 2014, we received news that Clinical Research Services at the University of California, San Francisco would begin charging us over $1600 per patient per inpatient hospital stay effective July 1st. This put additional pressure on us to delay data entry and analysis and instead focus our efforts on enrolling as many participants into the study before this deadline to make the best use of our remaining funds. Due to these drastically increased rates, we decided to end recruitment once we had dosed 203 participants rather than the proposed goal of 216 (our protocol allowed for us to dose up to 216 participants so we would have 200 participants with useable data).

  In early March, the nurse practitioner who completed our physical assessments and blood draws to medically clear participants for this study left his position at the VA with short notice. The interim nurse practitioner had to learn a large number of study protocols for many studies at the SFVAMC and was only scheduled to work two days per week. This resulted in a number of minor delays, but luckily did not pose a significant barrier.

  We also experienced delays with data entry and data cleaning, particularly with regard to our sleep data, due to our prioritizing subject enrollment in Y5 Q4.

  In response to all of these challenges, during Year 5 we applied for and were granted a No-Cost Extension to complete this study.

- **Recruitment and Enrollment Challenges**

  All study procedures are now complete. Enrollment greatly improved in Y5. We targeted recruitment of students in the health professions at nearby universities, and found that generally, this population was interested in the hypotheses of this study, eligible for participation and had reliable attendance at study appointments. These students so enjoyed participation in our study that word of mouth spread quickly, and we had to turn away some interested participants because our hospital admission schedule was fully booked. These recruitment improvements enabled us to meet our target enrollment before the newly instated hospital admission charges were in effect.

Subtask #6: Score and Analyze Data

A study database has been built, validated and tested and edit checks to the database have been programmed and validated. All neurocognitive data has been scored by trained and qualified study staff, and 100% of this data has been QC'd by our study psychologist.

Historically, study data has been scored and cleaned on an ongoing basis in hopes of shortening the cleaning and analysis timelines required after enrollment. However, due to our increased enrollment efforts in Y5, we are behind on some of the data scoring and cleaning. We are currently in the midst of addressing this.

During Y6, we will complete data scoring, data cleaning and complete our internal QC efforts. We will complete our visits with an externally contracted monitor to ensure data integrity and upon ensuring the data is cleaned, we will lock the database, open the blind and begin data analysis. We will analyze study data and work on the writing and submission of final reports and manuscripts.

Other Accomplishments Completed During Year 5:

- Reporting:
  Ongoing reports have been submitted as follows:
  - Safety listings were submitted to Actelion Pharmaceuticals on a monthly basis.
  - Progress reports were submitted to the Department of Defense on a quarterly basis and will continue through Y6.
  - Progress reports were submitted to the FDA on an annual basis and will continue through Y6.

- Human and Animal Study Collaboration:
  The San Francisco (human study) and SRI International (animal study) teams met either monthly or biweekly via teleconference throughout Year 4 to share progress updates, scientific rationale, and future planning initiatives. An in-person collaborative meeting was hosted by SRI in August 2011 and the most recent meeting was hosted at the SFVAMC in September 2012, just after the conclusion of Year 3. At each in-person meeting, members from each team gave presentations related to research rationale, progress, and future directions. The study teams have kept in regular contact via telephone conferencing, with our most recent discussion in May of 2014. We plan to have a final in person meeting once we have preliminary results to report.

Tasks 2 – 5: Please refer to the attached report from Dr. Kilduff (Appendix 2) which details the progress made in reference to the animal studies.

KEY RESEARCH ACCOMPLISHMENTS

Task 1 Accomplishments:

- Revised informed consent documents and recruitment materials have been approved by UCSF CHR.
- All new study personnel (Study Coordinator, Recruiter, and Research Assistant) have been hired and trained on the study protocol and procedures.

- 203 eligible participants have been identified through recruitment and screening efforts and have undergone dosing procedures.
- Study enrollment is now complete.
- Our study team is now working on data entry and data cleaning in hopes of locking the database by the end of Y6 Q1.

Tasks 2 – 5 Accomplishments:
Please refer to the attached progress report from Dr. Kilduff (Appendix 2).

REPORTABLE OUTCOMES
Reportable outcomes related to Task 1 will not be available until later in Year 6. In keeping with our double-blind study design, we do not intend to open the blind on the human subjects study until all of our data on the 200 subjects is cleaned and the database is locked.

We expect to have preliminary data to report in Y6 Q1 and we look forward to presenting our findings in publications, research presentations and meeting presentations. Data Entry is ongoing and up to date. Screening data (demographics information, height, weight, pre-dosing hematology, urine toxicology, pregnancy test results and blood chemistry), baseline data (sleep habits and caffeine use), inpatient eligibility data (urine toxicology, pregnancy test results), inpatient testing data (neuropsychological testing, vital signs and symptoms checklist), and follow up data (blood chemistry, adverse events reporting) are all up to date in our database and ready to be analyzed. We are now working on scoring, entering and cleaning the data from participant sleep assessments and once we complete our internal QC efforts, our external monitoring and have locked the database we will be ready to begin analyses.

Reportable outcomes related to Tasks 2 – 5 are noted in the attached progress report from Dr. Kilduff (Appendix 2). In addition, the following collaborative paper is under review:

Morairty SR, Wilk A, Lincoln W, Neylan TC, Kilduff TS. The Hypocretin/Orexin Antagonist Almorexant Promotes Sleep Without Impairment of Performance in Rats. In submission (Frontiers in Neuroscience).

CONCLUSION
Preclinical data indicate that animals treated with almorexant are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The purpose of this research is to test related hypotheses in both animals and humans. Enrollment of human subjects began during Year 2 and was completed in Y5 Q4. The findings from the animal component of the study were consistent with the hypothesis that disfacilitation of wake-promoting systems by almorexant results in less functional impairment than the general inhibition of neural activity produced by zolpidem (Appendix 2).

APPENDICES
Appendix 1: Human Study Protocol

Appendix 2: Animal Studies Progress Report

Appendix 1: Human Study Protocol

Summary of Protocol Changes
NEY-1413 Protocol
Version 10 – January 30, 2014

1.) Procedure Changes

- Our modified consent document was approved on January 30, 2014. Effective this date, subjects were given the option of participating in an optional procedure to compare a commercially available sleep monitoring device to that of our gold standard actigraphy. This involved wearing a wristwatch-like device during the three day inpatient hospital stay. Subjects who agreed to participate in this optional procedure consented for the manufacturer (Basis) to have access to de-identified demographic information.

CLINICAL STUDY PROTOCOL

Title:	Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance
Protocol number:	NEY-1413
Protocol Version/Date:	Final Version 10.0 30 January 2014
Phase:	Investigator-Initiated
Investigational Drug:	Almorexant
Investigator-Sponsor:	Thomas C. Neylan, M.D. Northern California Institute for Research and Education 4150 Clement Street (116P) San Francisco, CA 94121
Medical Monitor:	Frank Schoenfeld, MD Northern California Institute for Research and Education 4150 Clement Street (116P) San Francisco, CA 94121
Study Sites:	University of California, San Francisco Clinical and Translational Sciences Institute Clinical Research Center 505 Parnassus Avenue San Francisco, CA 94143 San Francisco Department of Veterans Affairs Medical Center 4150 Clement Street San Francisco, CA 94121

This clinical study will be conducted in accordance with Standard Operating Procedures (SOPs), current Good Clinical Practice (GCP) and the provisions of International Conference on Harmonization (ICH) Guidelines

Protocol Approval
NEY-1413
Final Version 9.0 28 March 2013

Investigator-Sponsor		
	Thomas Neylan, MD	**Date**
Co-Investigator		
	Steven Batki, MD	**Date**
Co-Investigator		
	Kristin Samuelson, Ph.D.	**Date**
Biostatistician		
	Thomas Metzler, M.S.	**Date**
Study Coordinator		
	Terri Motraghi, M.Sc.	**Date**

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ABBREVIATIONS

AE	Adverse Event
AASM	American Academy of Sleep Medicine
BzRAs	Benzodiazepine Receptor Agonists
CRC	Clinical Research Center
CCRC	University of California, San Francisco Clinical Translational and Sciences Institute Inpatient Clinical Research Center
UCSF CHR	University of California, San Francisco Committee on Human Research
CNS	Central Nervous System
CPT	Conners' Continuous Performance Test II
CRF	Case Report Form
DMP	Data Management Plan
DS	Digit Span Subtest of the Wechsler Adult Intelligence Scale Fourth Edition
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders Fourth Edition Text Revision
EEG	Electroencephalogram
FDA	Food and Drug Administration
GABA	Gamma-Aminobutyric Acid
GCP	Good Clinical Practice
GP	Grooved Pegboard Motor Test
ICH	International Conference on Harmonisation
IND	Investigational New Drug Application
IQ	Intelligence Quotient
IRB	Institutional Review Board
MWT	Maintenance of Wakefulness Test
NREM	Non-Rapid Eye Movement
ORP HRPO	Federal Office of Research Protections Human Research Protection Office
P-A	Paired Associates Learning Task
PMDD	Premenstrual Dysphoric Disorder
PMS	Premenstrual Syndrome
PSG	Polysomnography
PSQI	Pittsburgh Sleep Quality Index
PSST	Premenstrual Symptoms Screening Tool
PVT	Psychomotor Vigilance Test
QC	Quality Control

R&D Committee	Veterans Affairs Research and Development Committee
REM	Rapid Eye Movement
RAVLT	Rey Auditory Verbal Learning Test
SAE	Serious Adverse Event
SC	Symptom Checklist
SCID	Structured Clinical Interview for DSM-IV TR Axis I Disorders
SFDVAMC	San Francisco Department of Veterans Affairs Medical Center
SSS	Stanford Sleepiness Scale
Stroop	Stroop Color-Word Test
Towers	Tower Test from Delis-Kaplan Executive Function System
USAMRMC	U.S. Army Medical Research Materiel Command
WAIS-IV	Wechsler Adult Intelligence Scale Fourth Edition
WASO	Wake after Sleep Onset
WMS	Wechsler Memory Scale

SYNOPSIS

Protocol Number:	NEY-1413
Study Title:	A Double-Blind, Placebo-Controlled, Randomized, Parallel-Group Study Comparing the Effect of a Novel Hypocretin/Orexin Antagonist (Almorexant) Versus a Standard Hypnotic (Zolpidem) and Placebo on Neurocognitive Performance
Number of Sites:	1
Treatment Duration:	One-time Dose
Study Duration:	10 days, with a follow-up visit within 5 – 12 days of dosing
Study Population:	216 healthy male and female volunteers
Rationale:	In recent years, there has been increased focus on neurocognitive effects of hypnotic medications that adversely affect behavior during unanticipated awakenings during the night. Concerns regarding untoward effects of hypnotics during the sleep period have led to a Food and Drug Administration (FDA) class warning for all hypnotic drugs. These concerns are particularly relevant to the personnel of the military and those in other professions who have an occupational risk of poor sleep and who are expected to perform without impairment upon awakening. Almorexant is a hypocretin/orexin antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with almorexant are easily aroused from sleep and behave free of ataxia and other impairment. If this observation is confirmed in humans, it will have substantial implications for the management of disturbed sleep in both military and civilian populations.
Study Objectives:	To compare neurocognitive performance at peak concentration at midpoint during the habitual wake period in subjects randomized to almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo.
Study Design:	The study will take place at the San Francisco Department of Veterans Affairs Medical Center (SFDVAMC) and the University of California, San Francisco Clinical Translational and Sciences Institute inpatient Clinical Research Center (CCRC). The study will involve healthy volunteers who are considered normal sleepers per the Research Diagnostic Criteria for Normal Sleepers and who are free of medical disorders and specified psychiatric disorders. After informed consent has been obtained and eligibility has been confirmed, subjects will be scheduled for the 10-day study period. During the first seven days of the study period (the sleep/wake

	monitoring period), subjects will be asked to maintain a sleep diary and wear a wrist activity monitor (actigraph) 24 hours per day. Subjects will be admitted to the CCRC on the eighth day of the study period, two days prior to study drug administration. Subjects' sleep will be monitored with polysomnography (PSG) during each night on the CCRC, and subjects will continue to maintain a sleep diary and wear an actigraph during the three-day hospital stay. Subjects will be randomized in a double-blind fashion to one of four groups (almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo). Study drug will be provided to a nurse on the CCRC by an unblinded research pharmacist. The nurse and all other study personnel will remain blinded when study drug is dispensed to subjects. Following dosing, subjects will be accompanied by study personnel and instructed to remain awake. Neurocognitive, objective alertness, and subjective symptom assessments will be administered for several hours following dosing. Adverse events (AEs) will be assessed at the time of admission to the CCRC and on each day of the subject's stay in the CCRC. Subjects will be debriefed and discharged from CCRC on the morning of the fourth day on the unit. They will be required to return to the CRC at the SFDVAMC within 5 – 12 days of dosing for a safety lab test (liver function).
Inclusion Criteria:	1.) Male and female subjects between the ages of 19 and 39 determined to be physically healthy by physical exam and laboratory assessments; 2.) Habitual wake time between 0600 hr and 0900 hr maintained within the past month; 3.) Habitual bedtime between 2200 hr and 0100 hr maintained within the past month; 4.) Body Mass Index (BMI) >18 and < 28 kg/m^2; 5.) Ability to communicate well with the Investigator and to understand the study requirements.
Exclusion Criteria:	1.) Diagnosis of a sleep disorder within two years of screening or current sleep disturbance as suggested by a global score of > 5 on the Pittsburgh Sleep Quality Index (PSQI); 2.) Current presence of two or more risk categories on the Berlin Questionnaire for sleep apnea and overnight oximetry showing 10 desaturation events per hour or other results which are, in the judgment of the Investigator-Sponsor, suggestive of sleep apnea. 3.) A current or lifetime diagnosis of any psychiatric disorder with psychotic features, major depression, bipolar disorder, panic disorder, obsessive-compulsive disorder, posttraumatic stress disorder, generalized anxiety disorder, dysthymia, or agoraphobia without panic disorder, or current diagnosis of

	depressive disorder not otherwise specified, assessed using the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) Axis I Disorders (SCID); 4.) A current diagnosis of alcohol or substance abuse or dependence or a history of alcohol or substance abuse or dependence within the past year, assessed using the SCID; 5.) Subjects who are pregnant, lactating, or planning to become pregnant or subjects who are not willing to use an acceptable form of birth control during the study; 6.) Lifetime history of brain injury (including concussions, mild traumatic brain injuries, or loss of consciousness for ≥ 10 minutes which resulted in the development of persistent symptoms lasting ≥ 1 month), stroke, brain hemorrhage, seizures (not including infantile febrile seizures), epilepsy, or brain infection caused by meningitis, encephalitis, or any other infectious agent. 7.) Systemic illness affecting central nervous system (CNS) function; 8.) Cardiovascular disease (to include but not limited to arrhythmias, valvular heart disease, congestive heart failure, history of myocardial infarction or family history of sudden cardiac death), hypertension, or hypercholesterolemia; 9.) Asthma or other reactive airway diseases; 10.) Hepatic impairment (Child-Pugh A, B, C); 11.) Any other chronic or unstable medical conditions; 12.) Current use of statins, ketoconazole, prescription or over-the-counter medications or herbal supplements containing psychoactive properties or stimulants in the judgment of the Investigator-Sponsor or Medical Monitor; 13.) Treatment with another investigational drug; 14.) Current daily use of any other medication unless specifically approved by the Investigator-Sponsor; 15.) Consumption of grapefruit (including grapefruit juice) or treatment with moderate or strong inhibitors of cytochrome P450 3A4 (CYP3A4) within one week prior to randomization; 16.) Treatment with drugs metabolized by CYP2D6 isoenzyme with a narrow therapeutic index within one week prior to randomization; 17.) Self-reported regular nicotine use within the past 30 days involving > 4 cigarettes per week or > 2 cigarettes per day; 18.) Self-reported consumption of alcohol within the past 30 days of >14 standard drinks per week or ≥ 5 standard drinks on any day (men), or > 7 standard drinks per week or ≥ 4 standard drinks on any day (women).

	19.) Use of opioids, benzodiazepines, amphetamines, cocaine, cannabis, or any other illicit drugs within 30 days of screening by self report or a urine toxicology screen; 20.) Known liver disease or abnormal liver function tests assessed at the time of screening; 21.) Self-reported regular caffeine use in excess of 400 mg per day on average within six months of screening; 22.) Habitual long sleepers (> 9 hours) or short sleepers (< 5 hours); 23.) Shift work within one month prior to the screening visit or planned shift work during the study; 24.) Subjects who have traveled > 3 time zones within one week prior to the screening visit or any other visit; 25.) Known hypersensitivity or contraindication to any excipients of the drug formulation.
Outcome Measures:	<u>Primary Endpoints:</u> 1.) A comparison between groups on performance on the following neurocognitive measures: Rey Auditory Verbal Learning Test (RAVLT), Digit Span subtest of the Wechsler Adult Intelligence Scale IV (DS), Grooved Pegboard motor test, Paired-Associates subtest of the Wechsler Memory Scale (P-A), Stroop Color-Word Test (Stroop), Tower Test from Delis-Kaplan Executive Function System (D-KEFS Tower), Psychomotor Vigilance Test (PVT), and Conners' Continuous Performance Test II (CPT). 2.) A comparison between groups on latency to sleep onset measured by Maintenance of Wakefulness Tests (MWT) at 30 minutes and 150 minutes post-dose. 3.) A comparison between groups on low frequency EEG power during artifact free wake time as measured during MWTs. <u>Secondary Endpoints:</u> 1.) A comparison between groups on latency to sleep onset measured by MWTs at 270 and 390 minutes post-dose. 2.) A comparison between groups on Stanford Sleepiness Scale (SSS) scores. <u>Covariates:</u> 1.) Polysomnography (PSG) – Total Sleep Time on the night prior to the day of dosing. 2.) Actigraphy – Average sleep duration.
Statistical Considerations:	It is hypothesized that subjects receiving zolpidem 10mg will show greater impairment in neurocognitive performance compared to subjects receiving placebo, almorexant 100mg, or almorexant

	200mg. This hypothesis will be tested by comparing groups on post-medication performance tests using pre-medication test scores as covariates. Where multiple administrations of a performance test are given either pre-or post-medication, mixed effects models will be used, with the group by time (i.e., pre- vs. post-medication) interaction effect serving as the test of the hypothesis. Where a test is administered only once pre- and post-medication, the statistical test will be a one-way ANCOVA comparing mean scores on the four groups, with the pre-medication test score serving as the covariate. Planned comparisons will be conducted to compare the zolpidem 10mg group with placebo, almorexant 100mg, and almorexant 200mg separately. P-value adjustments will be made for multiple endpoint variables within any given neurocognitive domain using a step-down non-parametric re-sampling-based procedure. Primary analyses will be intent-to-treat, including all subjects randomized regardless or dropout or missing data status. Missing data will be carefully characterized and multiply imputed if necessary.

1. INTRODUCTION

1.1 Background

In recent years, there has been increased focus on cognitive side effects associated with sleep-inducing medications that may contribute to unusual behavior during unexpected awakenings during the night. Concerns regarding these side effects have led to a Food and Drug Administration (FDA) class warning for all sleep-inducing medication. These concerns are particularly important to the military and other professions that have an occupational risk of poor sleep and being unexpectedly awakened with an expectation to perform without impairment.

Almorexant is a hypocretin/orexin antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Hypocretin/orexin is a neuropeptide system that stimulates arousal and is involved in sleep regulation. Disruption of the hypocretin/orexin system has been shown to result in the sleep disorder narcolepsy in both animals and humans, indicating that this system is part of the intricate sleep/wakefulness regulatory network. Hypocretin receptors are found in many brain regions, although receptor expression is weak in the cortex and high in brain regions associated with arousal state regulation, particularly the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems. Since the hypocretin peptides are excitatory throughout the brain, hypocretin antagonists work by blocking this excitation rather than producing a generalized inhibition. To the contrary, benzodiazepine receptor agonists (BzRAs) such as zolpidem affect gamma-aminobutyric acid ($GABA_A$) receptors which have widespread distribution in the central nervous system (CNS), particularly in the cerebral cortex. BzRAs therefore cause a general inhibition of neural activity (2).

1.1.1 Preclinical Background

Preclinical data demonstrate that almorexant produces a profile that is unique among currently marketed hypnotic medications. For example, preliminary study results in rats treated with one of three doses (10mg/kg, 30mg/kg, and 100mg/kg) of almorexant, zolpidem or placebo in the middle of the dark active period (six hours after lights offset) demonstrated that the 30mg/kg and 100mg/kg doses of almorexant and zolpidem increased non-rapid eye movement (NREM) sleep for several hours after dosing, whereas 10mg/kg of almorexant had a more transient effect. All three doses of almorexant increased rapid eye movement (REM) sleep while REM was suppressed by zolpidem. Consequently, the REM-NREM ratio was unchanged relative to vehicle in animals treated with almorexant, but zolpidem produced a decreased REM-NREM ratio which is characteristic of BzRAs. When cumulative effects were assessed over the entire six-hour post-treatment period, it was evident that almorexant produced a dose-dependent decrease of wake and a dose-dependent increase in both NREM and REM sleep. This profile of a proportional increase of REM and NREM sleep appears to be unique among currently marketed hypnotic medications (3).

Additionally, almorexant appears to have few side-effects on regulated physiological systems. Preliminary studies comparing the effects of varying doses of almorexant, zolpidem, and placebo on core body temperature in rats revealed that zolpidem-treated animals experienced a significant and prolonged change in core body temperature post-

treatment, but there was relatively little change in core body temperature associated with any dose of almorexant (3).

In studies involving somnolent rats treated with almorexant, the rats showed an immediate reversibility of the hypnotic effect with no impairment on motor performance tasks (3). If similar observations are confirmed in humans, there will be enormous implications for the management of disturbed sleep in both military and civilian populations.

1.1.2 Clinical Background

Because hypocretins are implicated in coordinating states of wakeful vigilance, there has been a rapid development of small molecule hypocretin 1 and hypocretin 2 antagonists for possible use in insomnia. At present, there are robust drug discovery programs for hypocretin1/hypocretin 2 antagonists sponsored by Actelion, Glaxo-Smith Kline, Merck, Banyu, Sanofi-Aventis, and Janssen. In 2007, Actelion presented results of a multi-site, double-blind placebo controlled trial in insomnia patients examining the effects of 50mg, 100mg, 200mg, and 400mg doses of almorexant at bedtime. The results showed significant improvement in sleep efficiency and reduced wake after sleep onset (WASO) at doses of 100mg and higher (4). There was no occurrence of cataplexy at any of the dosages used. Almorexant has an elimination half-life of 1.4 hours and effects on sleep electroencephalography (EEG) were absent after 6.5 hours (3).

Almorexant was well-tolerated in studies completed to date, including nineteen Phase I studies in healthy and hepatically impaired subjects, two dose-finding studies in adult and elderly patients with primary insomnia, and one Phase III study in primary insomnia. 519 healthy and hepatically impaired subjects were exposed to at least one dose of almorexant in Phase I studies. 633 subjects with primary insomnia have been exposed to at least one dose of almorexant in completed studies. Maximum exposure was up to 400mg daily for 1 day or up to 200mg for 16 days. 166 patients with primary insomnia received 200mg for at least 14 days, and 176 received 100mg for at least 14 days. The most frequently reported adverse events with almorexant were headache, fatigue, dizziness, and somnolence (40).

1.2 Rationale

At appropriate doses, all currently available FDA-approved prescription sleep-inducing agents induce restorative sleep. However, they also exert substantial performance-impairing effects at peak concentration in multiple domains of neurocognitive function. For example, multiple studies have shown impairment in driving within the six-hour window after ingesting zolpidem (6, 7). Other studies have documented impairment in balance and postural tone within two hours of taking zolpidem (8). Furthermore, there is solid evidence that at peak concentration, currently available sleep-inducing agents significantly impair the ability to consolidate new memories (9-12). This evidence therefore precludes the use of sleep-inducing agents under operational conditions in which individuals might be called upon to perform without impairment after taking the agent, which is particularly relevant to populations involved in military combat. Further,

there is an enormous accumulation of data linking disturbed sleep to a wide range of outcomes including daytime fatigue (13-15), impaired concentration and attention (16-19), increased risk for accidents and injuries (20, 21), worsened quality of life (22), increased aggression (23-26), and increased use of alcohol (27, 28). Several studies have also demonstrated that disturbed sleep is a potent risk factor for later onset development of major depression, panic disorder, alcohol, and substance abuse (27-30). Therefore, an effective treatment for sleep disturbances that can be safely utilized in deployed military personnel in combat operations without performance-impairing effects has the potential for improving the success of combat operations, inoculating soldiers against battlefield stress-related psychiatric illnesses, and preserving the psychological health of the soldiers throughout the full deployment lifecycle. The availability of such a treatment would also have a positive impact on the overall quality of life, physical, and psychological well being of the civilian population.

The study discussed in this protocol will involve a double-blind, placebo-controlled, randomized, parallel-groups study design and will involve a one-time oral administration of one of four dosing options to healthy volunteers: almorexant 100mg, almorexant 200mg, zolpidem 10mg, and placebo. These dosages have demonstrated favorable safety profiles in clinical trials (5). Subjects will be dosed at the average midpoint of the habitual wake period. Neurocognitive performance assessments will be administered at the time of peak plasma concentration. The study will establish whether almorexant is superior to zolpidem and placebo regarding neurocognitive performance at the estimated peak plasma concentration.

2. CLINICAL STUDY OBJECTIVES

2.1 Primary Objectives

Primary endpoints are listed below:

1.) A comparison between groups on performance on the following neurocognitive measures: Rey Auditory Verbal Learning Test (RAVLT), Digit Span subtest of the Wechsler Adult Intelligence Scale IV (DS), Grooved Pegboard motor test (GP), Paired-Associates subtest of the Wechsler Memory Scale (P-A), Stroop Color-Word Test (Stroop), Tower Test from Delis-Kaplan Executive Function System (D-KEFS Tower), Psychomotor Vigilance Test (PVT), and Conners' Continuous Performance Test II (CPT).
2.) A comparison between groups on latency to sleep onset measured by Maintenance of Wakefulness Tests (MWT) at 30 minutes and 150 minutes post-dose.
3.) A comparison between groups on low frequency EEG power during artifact free wake time as measured during MWTs.

2.2 Secondary Objectives

Secondary endpoints are listed below:

1.) A comparison between dosing groups on latency to sleep onset measured by MWTs at 270 and 390 minutes post-dose.
2.) A comparison between dosing groups on Stanford Sleepiness Scale (SSS) scores.

The following outcomes will be analyzed as covariates:
1.) Polysomnography (PSG) - Total Sleep Time on the night prior to the day of dosing.
2.) Actigraphy – Average sleep duration.

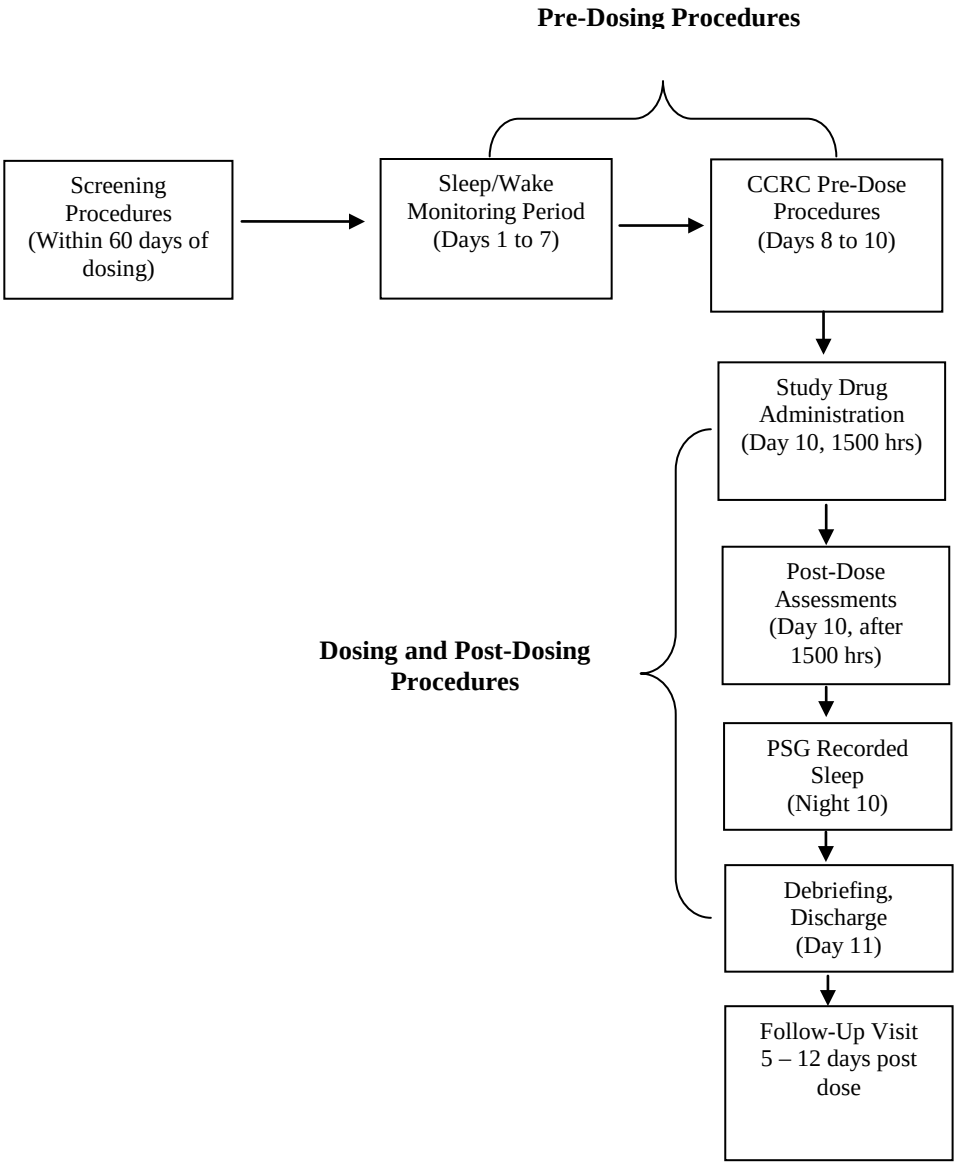
3. STUDY DESIGN

The study will take place at the San Francisco Department of Veterans Affairs Medical Center (SFDVAMC) and the University of California, San Francisco Clinical Translational and Sciences Institute inpatient Clinical Research Center (CCRC). The study will involve healthy volunteers who are considered normal sleepers per the Research Diagnostic Criteria for Normal Sleepers (1) as listed below:

1.) Subject has no complaints of sleep disturbance or daytime symptoms attributable to unsatisfactory sleep.
2.) Subject has a routine sleep/wake schedule characterized by regular bedtimes and rising times.
3.) There is no evidence of a sleep-disruptive medical or mental disorder.
4.) There is no evidence of sleep disruption due to a substance exposure, use, abuse, or withdrawal.
5.) There is no evidence of a primary sleep disorder.

Subjects will also be free of medical disorders and specified psychiatric disorders. After informed consent has been obtained and eligibility has been confirmed, subjects will be instrumented with wrist actigraphs to record their sleep/wake patterns for seven days; subjects will also be asked to complete a sleep diary during this one-week time period. Subjects will be admitted to the CCRC on the day after completion of the one-week sleep/wake monitoring period and two days prior to drug administration. Subjects' sleep will be monitored with PSG during each night at the CCRC, and sleep apnea will be screened for during the first night of PSG. Subjects will continue to maintain a sleep diary and wear an actigraph while at the CCRC. Subjects will be randomized in a double-blind fashion to one of four groups (almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo). An unblinded research pharmacist will provide study drug to a nurse at the CCRC for dispensing. The nurse and all other study personnel will remain blinded when study drug is dispensed to subjects. Following dosing, subjects will be accompanied by study personnel and instructed to remain awake. Neurocognitive, objective alertness, and subjective symptom assessments will be administered at regular intervals for several hours following dosing. Adverse events (AEs) will be assessed at the time of admission to the CCRC and on each day of the subject's stay in the CCRC. Subjects will be debriefed and discharged from the CCRC during the morning of the fourth day on the unit. They will be required to return to the SFDVAMC within 5 – 12 days of dosing for a safety lab test (liver function).

3.1 Study Design Schematic



4. SUBJECT SELECTION

Medically healthy men and women ages 19-39 (N = 216) will be recruited from newspaper advertisements, web based postings, websites, and flyers posted in various university and community sites. The age range is restricted to an upper limit of 39 years as a result of research showing a change in middle-aged individuals (defined as 40+ years of age) in terms of total sleep time and other sleep parameters that can affect performance outcomes independent of sleep deprivation and/or drug administration, which could therefore introduce a substantial source of error variance into the study (31). Interested potential subjects will be contacted by the study recruiter. If potential subjects agree, a 15 – 30 minute phone discussion will take place to determine whether they might be a match for the study. If the phone conversation indicates that the potential subjects may be a match for the study and they are still interested, they will be scheduled to meet with the study coordinator or another qualified study team member in person at the SFDVAMC for informed consent and further eligibility procedures.

4.1 Subject Inclusion Criteria

Subjects must meet all inclusion criteria in order to be eligible for the study:

1.) Male and female subjects between the ages of 19 and 39 determined to be physically healthy by physical exam and laboratory assessments;
2.) Habitual wake time between 0600 hr and 0900 hr maintained within the past month;
3.) Habitual bedtime between 2200 hr and 0100 hr maintained within the past month;
4.) Body Mass Index (BMI) >18 and < 28 kg/m^2;
5.) Ability to communicate well with the Investigator and to understand the study requirements.

4.2 Subject Exclusion Criteria

Any of the following criteria will exclude the subject from entering the study:

1.) Diagnosis of a sleep disorder within two years of screening or current sleep disturbance as suggested by a global score of > 5 on the Pittsburgh Sleep Quality Index (PSQI) (43);
2.) Current presence of two or more risk categories on the Berlin Questionnaire (42) for sleep apnea and overnight oximetry showing 10 desaturation events per hour or other results which are, in the judgment of the Investigator-Sponsor, suggestive of sleep apnea.
3.) A current or lifetime diagnosis of any psychiatric disorder with psychotic features, major depression, bipolar disorder, panic disorder, obsessive-compulsive disorder, posttraumatic stress disorder, generalized anxiety disorder, dysthymia, or agoraphobia without panic disorder, or current diagnosis of depressive disorder not otherwise specified, assessed using the Structured Clinical Interview for DSM-IV TR Axis I Disorders (SCID) (41);

4.) A current diagnosis of alcohol or substance abuse or dependence or a history of alcohol or substance abuse or dependence within the past year, assessed using the SCID (41);
5.) Subjects who are pregnant, lactating, or planning to become pregnant or subjects who are not willing to use an acceptable form of birth control during the study;
6.) Lifetime history of brain injury (including concussions, mild traumatic brain injuries, or loss of consciousness for $\geq$ 10 minutes which resulted in the development of persistent symptoms lasting $\geq$ 1 month), stroke, brain hemorrhage, seizures (not including infantile febrile seizures), epilepsy, or brain infection caused by meningitis, encephalitis, or any other infectious agent.
7.) Systemic illness affecting central nervous system (CNS) function;
8.) Cardiovascular disease (to include but not limited to arrhythmias, valvular heart disease, congestive heart failure, myocardial infarction or family history of sudden cardiac death), hypertension, or hypercholesterolemia;
9.) Asthma or other reactive airway diseases;
10.) Hepatic impairment (Child-Pugh A, B, C);
11.) Any other chronic or unstable medical conditions;
12.) Current use of statins, ketoconazole, prescription or over-the-counter medications or herbal supplements containing psychoactive properties or stimulants in the judgment of the Investigator-Sponsor or Medical Monitor;
13.) Treatment with another investigational drug;
14.) Current daily use of any other medication unless specifically approved by the Investigator-Sponsor;
15.) Consumption of grapefruit (including grapefruit juice) or treatment with moderate or strong inhibitors of cytochrome P450 3A4 (CYP3A4) within one week prior to randomization;
16.) Treatment with drugs metabolized by CYP2D6 isoenzyme with a narrow therapeutic index within one week prior to randomization;
17.) Self-reported regular nicotine use within the past 30 days involving > 4 cigarettes per week or > 2 cigarettes per day;
18.) Self-reported consumption of alcohol within the past 30 days of >14 standard drinks per week or $\geq$ 5 standard drinks on any day (men), or > 7 standard drinks per week or $\geq$ 4 standard drinks on any day (women).
19.) Use of opioids, benzodiazepines, amphetamines, cocaine, cannabis, or any other illicit drugs within 30 days of screening by self report or a urine toxicology screen;
20.) Known liver disease or abnormal liver function tests assessed at the time of screening;
21.) Self-reported regular caffeine use in excess of 400 mg per day on average within six months of screening;
22.) Habitual long sleepers (> 9 hours) or short sleepers (< 5 hours);
23.) Shift work within one month prior to the screening visit or planned shift work during the study;
24.) Travel of > 3 time zones within one week prior to the screening visit or any other visit;

25.) Known hypersensitivity or contraindication to any excipients of the drug formulation.

5. STUDY DRUG HANDLING

5.1 Allocation to Dosing Groups

Subjects will be randomly assigned to one of four dosing groups in a 1:1:1:1 ratio: almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo. Randomization will be stratified based on gender and caffeine use. Subjects will dose one time on Study Day 10 at 1500 hrs according to their assigned dosing group.

Almorexant (100mg and 200mg) is currently being investigated in a comprehensive Phase III program. Results indicate that almorexant was well-tolerated in the initial Phase III study. Further Phase III studies to evaluate long-term efficacy and safety are in preparation (4).

Zolpidem 10mg is an imidazopyridine class sedative hypnotic which received original United States market approval under the brand name Ambien® in 1992.

5.2 Breaking the Blind

The blind will be maintained through study completion except for cases of breaking the blind due to emergency medical necessity. In situations in which the CCRC nursing staff or other study personnel determines that it might be necessary to break the blind, he/she will be instructed to contact the Investigator-Sponsor or Medical Monitor. If approval is granted by the Investigator-Sponsor or Medical Monitor, the CCRC nurse will be authorized to contact the research pharmacist at the CCRC. The research pharmacist will maintain a master randomization list and he/she or an authorized designee will be available to break the blind if necessary.

5.3 Dosing Adherence/Study Compliance

Since only one dose will be administered to subjects by a nurse at the CCRC, deviations from the scheduled dosing regimen are not anticipated.

During the sleep-wake monitoring period which will take place throughout the week prior to admission to the CCRC, subjects will be required to maintain regular wake times between 0600 hr and 0900 hr and bedtimes between 2200 hr and 0100 hr. Additionally, subjects will be asked to avoid recreational drug use, naps, the consumption of grapefruit or grapefruit juice, alcohol, and/or nicotine. Subjects will also be asked to maintain stable caffeine use and to avoid crossing more than three time zones. Actigraphs will be utilized to monitor the subjects' sleep-wake patterns and will therefore serve as a check for compliance with the prescribed sleep regimen. Subjects will maintain daily sleep diaries during the 10-day study period which will capture the following items: lights out and wake clock times, estimated sleep latency, wake time in minutes after sleep onset, rating

of sleep quality on a scale of 1-100, caffeine use, and atypical events. Actigraphy and sleep diary data will be reviewed upon admission to the CCRC to determine compliance with the required sleep/wake schedule. An additional urine toxicology screening will be administered at the time of admission to the CCRC to rule out recent recreational drug use, and females will receive a urine pregnancy test at this time.

5.4 Drug Supplies

5.4.1 Formulation and Packaging

Actelion Pharmaceuticals Ltd. will provide almorexant 100 mg tablets, zolpidem 10 mg capsules, and matching placebo tablets and capsules. A double dummy design will be employed which will result in each subject receiving two tablets and one capsule. Study drug will be provided in bulk and will be shipped directly to the research pharmacy at the CCRC.

5.4.2 Preparing and Dispensing

The research pharmacist in the CCRC will maintain a copy of the randomization schedule and will receive the subject's randomization assignment at the time of hospital admission. The research pharmacist will dispense the assigned study drug to the nurse who will be administering the drug to the randomized subject.

5.4.3 Drug Administration

After obtaining the appropriate study drug from the research pharmacy, a CCRC nurse will administer the drug to the subject.

5.5 Drug Storage and Accountability

All drug products will be stored at the recommended temperature (room temperature at a maximum of 25^{o}C). Site personnel and study monitors will perform regular checks to document that the study drug is stored appropriately and is within the defined expiration period at all times. A drug accountability log will be completed by the research pharmacist when study drug is received and dispensed to subjects. Any unused drug will be destroyed at the conclusion of the study.

5.6 Concomitant Medications

Medication use will be assessed at screening. Concomitant medications will also be assessed when the subject arrives at the CCRC on Day 8, on each subsequent day in the CCRC (Days 9, 10, and 11), and at follow-up. All concomitant medications will be recorded in the source documents and transcribed onto the Case Report Forms (CRFs).

5.6.1 Disallowed Concomitant Medications and Dietary Restrictions

Use of statins, prescription or over-the-counter medications containing psychoactive properties or stimulants is exclusionary and is also prohibited during the study period. Subjects will be required to maintain stable caffeine consumption of 200 mg per day or less during the study. Alcohol, recreational drug, and nicotine use is prohibited during the 10 day study period. Consumption of grapefruit (including grapefruit juice) or treatment with moderate or strong inhibitors of cytochrome P450 3A4 (CYP3A4) within one week prior to randomization is prohibited.

6. STUDY PROCEDURES

6.1 Pre-Dosing Procedures

Screening

The study coordinator or another qualified, trained study team member will obtain informed consent from each potential subject prior to the initiation of eligibility procedures. During the informed consent meeting, the study will be explained and the subject's questions will be answered. Subjects will be allowed to take as much time as they need to make a decision and will be given the option of discussing their decision with their family, friends, or other healthcare providers.

- Physical Exam, Medical History, and Prior/Concomitant Medications Assessment (performed by a nurse practitioner at the SFVAMC CRC).
- Laboratory Analysis of Blood and Urine Samples: A urine sample and approximately 20ccs of blood will be collected for laboratory tests which will include a serum chemistry panel, liver function tests (including albumin), thyroid function tests, prothrombin time, complete blood count and differential, urine toxicology screen, and a urine pregnancy test (in women of childbearing potential). If lab values are out of range, subjects may be asked to repeat the blood draw for a retest to confirm their medical health.
- Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders (SCID [41]), performed by a trained mental health clinician
- Self-report Berlin Questionnaire (42) to determine likelihood of sleep disordered breathing. If subjects have two or more positive scoring categories, they will also be monitored with pulse oximetry.
- Self-report Pittsburgh Sleep Quality Index (PSQI [43])
- The Premenstrual Symptoms Screening Tool (PSST) to determine if female participants meet criteria for Premenstrual Dysphoric Disorder (PMDD) and/or moderate to severe PMS. For all females who meet criteria for PMDD and/or moderate to severe PMS, Days 8-10 will be scheduled to coincide with the follicular phase of the menstrual cycle (PSST [54]).
- Review of Inclusion/Exclusion Criteria

All screening assessments will be performed at the SFDVAMC, including the collection of blood and urine samples and laboratory analysis. Dosing with study drug must take

place within 60 days of when the screening assessments were administered. Screening assessments which were administered > 60 days prior to scheduled dosing will have to be repeated before subjects will be allowed to dose with study drug.
During the screening period the Vocabulary Subtest of the Wechsler Adult Intelligence Scale, Fourth Edition (WAIS-IV [48]) will be administered for the purpose of obtaining an IQ measure to ensure that all dosing groups are matched on intelligence. Vocabulary Subtest results will not be used to determine eligibility.

Sleep/Wake Monitoring (Days 1 to 7)

A seven-day sleep/wake baseline monitoring period will be scheduled for subjects who meet all inclusion and exclusion criteria. For all females who meet criteria for PMDD and/or moderate to severe PMS, Days 8-10 will be scheduled to coincide with the follicular phase of the menstrual cycle. Prior to the start of the baseline week, a practice version of the PVT will be administered. Subjects will be asked to wear wrist actigraphs 24 hours per day on each day of the seven day monitoring period, and they will also be asked to abide by the following instructions:

- Adhere to a consistent wake schedule of 0600 hrs – 0900 hrs and a lights out schedule of 2200 hrs – 0100 hrs.
- Avoid nicotine and recreational drug use.
- Maintain stable caffeine consumption of ≤ 400 mg per day.
- Avoid alcohol use. (Although subjects will be encouraged to avoid alcohol entirely, acceptable use is ≤ 14 drinks per week or < 5 drinks on any day for men, and ≤ 7 drinks per week or <4 drinks on any day for women.)
- Avoid the consumption of grapefruit or grapefruit juice.
- Avoid travelling > 3 time zones.
- Avoid naps.
- Avoid starting new medications unless they become necessary in the opinion of a physician.
- Use an acceptable form of birth control.

Subjects will maintain daily sleep diaries during the sleep/wake monitoring period which will capture the following data points: lights out and wake clock times, estimated sleep latency, wake time in minutes after sleep onset, rating of sleep quality on a scale of 1-100, caffeine use, and atypical events.

Day 8 (CCRC Admission)

Subjects will enter the CCRC in the evening and a urine toxicology screen will be performed. A urine pregnancy test will be performed for female subjects of childbearing potential. All subjects will be asked to report concomitant medications and AEs dating back to informed consent. Sleep diary data will be reviewed to determine compliance with the required sleep/wake regimen. Compliance with other study-related instructions will also be assessed at this time. While at the CCRC, subjects will receive a prescribed lights out time which will be consistent with the lights out regimen that was followed

during the baseline week. All subjects will be prescribed a 0700hr wake time during their stay at the CCRC. Subjects will have the choice of opting into an additional procedure whereby they wear a commercially available sleep monitoring device which we will compare to our gold standard actigraphy.

Subjects will continue to maintain a daily sleep diary and wear an actigraph during their stay at the CCRC. Additionally, during each night at the CCRC, subjects will have their sleep monitored with ambulatory PSG. Subjects will also be screened for obstructive sleep apnea which will involve thermistor measurements, pulse oximetry for detection of oxygen desaturation events, and two channels of respiratory inductive plethysmography to measure chest and abdominal movement during breathing. Subjects with an apnea/hypopnea index ≥ 10 will be excluded from the data analysis.

Day 9

Subjects will be awakened at 0700 hrs and will remain in the CCRC for monitoring. Caffeine consumption should remain consistent with what the subject consumed throughout the sleep/wake monitoring period, and caffeine will not be allowed after 1330 hrs. Naps will be prohibited. During the evening (prior to lights out), AEs and concomitant medications will be assessed. Subjects will have their sleep monitored with PSG.

Day 10 (Pre-Dose; 0700hrs – 1500hrs)

Subjects will be awakened at 0700 hrs. Caffeine consumption will remain consistent with what the subject consumed throughout the sleep/wake monitoring period, and caffeine will not be allowed after 1330 hrs. Beginning at 1000 hrs, subjects will be administered a series of baseline (pre-dose) neurocognitive assessments, objective alertness assessments, and subjective assessments. All assessments will be administered by qualified, trained, research technicians. Assessments to be administered are described below:

Stanford Sleepiness Scale: Subjects will be asked to rate themselves along a 7-point scale ranging from 1 (fully alert) to 7 (extremely sleepy). This scale will be administered just prior to each administration of the MWT. Administration time is less than 5 minutes.

Maintenance of Wakefulness Test: Subjects will be placed in a dimly lit room where they will sit comfortably and receive instruction to keep their eyes open and attempt to remain awake while being monitored via standard MWT EEG leads. If the subject falls asleep, he/she will be awakened after three epochs of sleep as determined by EEG trace. Administration time is 20 minutes.

Psychomotor Vigilance Test: Subjects will be required to press a button each time a target is presented. Administration time is 10 minutes.

Rey Auditory Verbal Learning Test – List 1: Each subject will be read a list of 15 words and asked to repeat back as many as they can remember. The task is repeated 4 more

times. Subsequently, a new interference list is read and the subject is asked to repeat back items from that list. Then the subject is asked to recall items from the original list. Administration time is approximately 10 minutes.

Continuous Performance Test II: Subjects will be required to press the space bar or click the mouse button when any letter except for the target letter "X" appears. Administration time is 15 minutes.

Symptom Checklist: Subjects will be asked if they are experiencing specific symptoms commonly associated with hypnotics. If they endorse any of the symptoms on the checklist, they will be asked whether the symptoms are mild, moderate, or severe. Administration time is approximately 5 minutes.

Vital signs (sitting blood pressure and heart rate) will be obtained several times throughout Day 10. Staff will also query for AEs at these time points.

6.2 Study Dosing

Day 10 (Dosing and Post-Dose, 1500hrs - 2200hrs)

Subjects will dose at 1500 hrs. Shortly after dosing, a PVT administration will take place. MWTs (preceded by the SSS each time) will be administered at 1530 hrs, 1730 hrs, 1930 hrs, and 2130 hrs.

Based on the literature (3), it is estimated that almorexant will reach peak blood concentration between 1600 hrs and 1800 hrs. Around this timeframe, subjects will be administered the PVT, CPT, and SC, in addition to the MWT and SSS. The following neurocognitive assessments will also be administered during this timeframe:

Paired-Associates Learning Task: Subjects will be read 10 pairs of words. They will then be read, in a different order, the first word from each pair for which they are to recall the associated second word. The list will be presented and followed by recall two more times (with pairs in a different order each time). The first administration of the Paired-Associates Learning Task (given during the timeframe of 1600hrs – 1800hrs) will test immediate recall, during which errors are corrected. The test will be administered again several hours after the first administration using the same word list to assess delayed recall. Errors will not be corrected during the delayed recall trial.

Rey Auditory Verbal Learning Test – List 2: The RAVLT will be administered again during the 1600 – 1800hrs timeframe, but with a new list.

Grooved Pegboard Test: The test consists of 25 holes with randomly positioned slots. Pegs with a key along one side must be rotated to match the hole before they can be inserted and subjects must place the pegs in the holes as quickly as possible. Administration time is approximately 10 minutes.

Stroop Color-Word Test: Subjects will be given three sheets of paper, one at a time. The Word page consists of the words "red," "green," and "blue" printed randomly in rows in black ink. Subjects will be asked to read as many words as they can out loud in a 45 second time period. The Color page consists of 100 items, all written as "XXXX," printed in either green, red, or blue ink. Subjects will be asked to name as many colors as they can out loud in a 45 second time period. The Color-Word page consists of the words from the Word page printed in the colors from the Color page. The words and the colors they are printed in do not match one another. Subjects will be asked to name as many colors as they can in a 45 second time period. Total administration time is approximately 10 minutes.

Tower Test from Delis-Kaplan Executive Function System: Subjects will be asked to complete problem-solving tasks which will involve moving disks on pegs to match an arrangement shown to them in a picture. Administration time is approximately 20 minutes.

Digit Span: Subjects will be read a sequence of digits and asked to repeat the digits in the same sequence. For the second portion of the test, subjects will be read a sequence of digits and asked to repeat the digits in reverse order. For the third portion of the test, subjects will be read a sequence of digits and asked to repeat the digits in order from the lowest number to the highest. Administration time is approximately 6 minutes.

After the time window of 1600 hrs - 1800 hrs, subjects will receive additional administrations of the PVT, SC, and RAVLT (third list). Two more MWT administrations will also take place. The final assessment will begin at 2130 hrs.

Study personnel will remain with the subjects throughout testing and subjects will be kept awake until all assessments have been completed. Some of the neurocognitive tests will be audio recorded for quality control purposes.

Night 10 (Post-Dose)

AEs will be assessed prior to the prescribed lights out time. Subjects will engage in undisturbed, PSG recorded sleep.

Day 11 (Discharge)

Upon awakening at 0700 hrs, subjects will have all electrodes removed and will be debriefed prior to being discharged from the CCRC. AEs will be assessed prior to discharge.

Safety Follow-Up

Within 5 – 12 days of dosing with study drug, subjects will be required to have a blood draw performed for a liver function test. This procedure will be performed at the SFDVAMC. Approximately 5ccs of blood will be drawn and analyzed at the SFDVAMC laboratory. If lab values are out of range, the subject may be asked to repeate the blood draw for a retest. The occurrence of AEs and concomitant medications since the day of discharge will be assessed.

7. STUDY OUTCOMES AND SAFETY ASSESSMENTS

7.1 Study Outcome Assessment Measures

A description of the measures which will be utilized for the outcome analyses is provided below:

Psychomotor Vigilance Test: The PVT is a widely used instrument that measures sustained attention and reaction time (49). Extensive work with this measure has demonstrated that the PVT is not affected by practice effects and is a highly sensitive measure of the effects of disrupted circadian rhythms from shift work (17) and chronic sleep deprivation (18, 19). PVT-192® devices will be utilized for this study. The PVT has a random inter-stimulus interval of 2-10 seconds and can be collected over a 10 minute period. The main measure will be performance lapses (reaction time > 500 ms) per 10 minute period. Secondary measures will include total time of lapses, frequency of false responses, frequency of non-responses, durations of the 10% fastest and 10% slowest responses, and performance decrement across time on the task.

Stanford Sleepiness Scale: The SSS is a subjective measure of sleepiness in which subjects rate themselves along a 7-point scale ranging from 1 (fully alert) to 7 (extremely sleepy) (50). Subjective sleepiness ratings will be collected in order to verify the sedative effects of zolpidem and the two doses of almorexant.

Maintenance of Wakefulness Test: The MWT is widely used to demonstrate significant pre and post treatment differences in excessive sleepiness. Sleep onset is defined as the first occurrence of > 15 seconds of cumulative sleep in a 30 second epoch. Latency to the first 30 seconds of sleep will be scored online by the attending sleep technologist. The subject will be awakened within 90 seconds of falling asleep.

Rey Auditory Verbal Learning Test: The RAVLT is a word learning task and a measure of short-term auditory memory and learning, as well as delayed auditory memory (52, 53).

Grooved Pegboard Test: A measure of manipulative dexterity, this test requires complex visual-motor coordination (51).

Paired-Associates Learning Task: This associative learning sub-test of the Wechsler Memory Scale tests the ability to learn and recall pairs of words, some of which are related (e.g., north/south) and others which are unrelated (e.g., eagle/jury) (47).

Immediate and delayed recall trials will be scored for the number of correctly recalled pairs.

Continuous Performance Test II: The CPT assesses attention and working memory as well as executive function (44). Specifically, the CPT measures response inhibition via commissions (an aspect of executive function) and sustained attention via omissions. There is evidence in the literature which suggests that continuous performance tasks are sensitive to sleep-inducing agents (34). Scores will be based on response time and errors, inclusive of omissions and commissions.

Stroop Color-Word Test: The Stroop is a widely used putative measure of executive function that measures response inhibition (35). The Color-Word score will be computed, which measures the subject's ability to inhibit or override the tendency to produce the more automatic or dominant response (i.e., to name the color word rather than the color).

Tower Test from Delis-Kaplan Executive Function System: D-KEFS Tower is typically used for the assessment of executive function, specifically to detect deficits in planning, decision making, and problem solving (45). Literature provides evidence of a link between performance on towers tasks and sleep (32).

Digit Span: Digit Span is a subtest of the WAIS-IV which measures attention and working memory and has been found to be sensitive to sleep-inducing agents (36, 48).

The following measures will serve as covariates:

Actigraphy: The primary actigraph measures are habitual sleep onset and offset times and the range of variability around these data points. The wrist actigraph provides continuous activity data using a battery-operated wristwatch-sized microprocessor that senses motion with an accelerometer. Subjects can also indicate lights on, lights off, and other salient events by pressing an event marker on the actigraphs. The actigraphs will be initialized with the ActMe program (Ambulatory Monitoring, Inc.) using the PIM sampling mode in one-minute epochs for conventional actigraphic sleep-wake estimation.

Polysomnography: The primary PSG measure is total sleep time on the night prior to the day of dosing and neurocognitive testing. PSG recordings will be obtained with ambulatory PSG and the parameters recorded will follow current guidelines as defined in the AASM Manual for the Scoring of Sleep and Associated Events (37).

The Embla Titanium ambulatory recorders record up to 34 channels. The sampling frequency ranges from 256Hz to 512 Hz. High and low frequency filters will be added while scoring the data manually and in spectral analysis. 60Hz notch filters may be applied to remove electrical noise. Raw files will be kept with only anti-aliasing filters. Spectral analysis will organize sleep epochs by stage and time. Artifacts will be tagged for removal for spectral analysis.

7.2 Safety Assessment Measures

Symptom Checklist: This checklist captures common symptoms experienced by subjects taking hypnotic medications. Reports of symptoms will be collected in order to compare possible drug side effects.

AEs will be assessed on a regular basis throughout the study and at the follow-up visit.

A liver function test will be performed on all subjects within 5 – 12 days of dosing with study drug.

8. ADVERSE EVENT REPORTING

8.1 Adverse Event Definitions

An AE is defined as any untoward medical occurrence that takes place in a clinical study, regardless of the causal relationship of the event with the investigational drug or study treatment(s). Any event occurring after the clinical trial participant has signed the study informed consent documentation should be recorded and reported as an AE.

An AE can be any unfavorable and unintended sign, symptom, or disease temporally associated with the use of the investigational product, whether or not considered related to the investigational product. A new condition or the worsening of a pre-existing condition will be considered an AE.

An abnormal test finding will be classified as an AE if one or more of the following criteria are met: a.) the test finding is accompanied by clinical symptoms; b.) the test finding necessitates additional diagnostic evaluation(s) or medical/surgical intervention, including significant additional concomitant drug or other therapy; c.) the test finding leads to discontinuation of subject participation in the clinical study; d.) the test finding is considered an AE by the Investigator-Sponsor of the IND application.

For each AE, the date and time of onset, a description of the event, severity, seriousness, action taken, relationship to the study drug, outcome, and date of resolution will be recorded.

A **Serious Adverse Event (SAE)** is defined as an AE that results in any of the following:

- Death
- Life-threatening event – An event in which the subject is at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.
- Requires hospitalization or prolongs existing inpatient hospitalization, not inclusive of a pre-planned elective hospital admission for treatment of a pre-existing condition that has not significantly worsened or a diagnostic procedure.
- Results in persistent or significant disability or incapacity.
- Results in congenital abnormality or birth defect.

- An important medical event occurs which requires medical intervention to prevent any of the above outcomes. Important medical events are those which may not be immediately life-threatening but may jeopardize the subject and may require intervention to prevent one of the serious outcomes listed above.

An **Unexpected Adverse Event** is defined as any AE in which the frequency, specificity, or severity is not consistent with the risk information described in the clinical protocol or elsewhere in the current IND application or Investigator's Brochure.

8.2 Recording Requirements

8.2.1 Eliciting Adverse Event Information

AEs will be assessed when subjects check into the CCRC and again during each evening at the CCRC. Additionally, subjects will complete a Symptom Checklist at various scheduled time points throughout the day of dosing and asked to report the occurrence of any other AEs.

8.2.2 Recording Requirements

All observed or volunteered adverse drug events (serious or non-serious) and abnormal test findings, regardless of treatment group or suspected causal relationship to the investigational drug or study treatment(s) will be recorded in the subjects' case histories. For all AEs, sufficient information will be pursued and/or obtained so as to permit a.) an adequate determination of the outcome of the event; and b.) an assessment of the causal relationship between the AE and the study drug.

AEs or abnormal test findings felt to be associated with the investigational drug or study treatment(s) will be followed until the event (or its sequelae) or the abnormal test finding resolves or stabilizes at a level acceptable to the Investigator-Sponsor.

8.3 Reporting of Adverse Events

8.3.1 Reporting of Adverse Events to the FDA

Written IND Safety Reports

The Investigator-Sponsor will submit a written IND Safety Report to the responsible new drug review division of the FDA for any observed or volunteered AE that is determined to be a.) associated with the investigational drug or study treatment(s); b.) serious; and c.) unexpected. Each IND Safety Report will be prominently labeled, "IND Safety Report."

Written IND Safety Reports will be submitted to the FDA as soon as possible and within 15 calendar days following the Investigator-Sponsor's receipt of the respective AE information. For each written IND Safety Report, the Investigator-Sponsor will identify

all previously submitted IND Safety Reports that addressed a similar AE experience and will provide an analysis of the significance of newly reported AE in light of the previous, similar report(s).

Follow-up information to an IND Safety Report will be submitted to the applicable review division of the FDA as soon as the relevant information is available. If the results of the Investigator-Sponsor's follow-up investigation show that an AE that was initially determined to not require a written IND Safety Report does, in fact, meet the requirements for reporting; the Investigator-Sponsor will submit a written IND Safety Report as soon as possible and within 15 calendar days after the determination was made.

In accordance with FDA requirements, annual safety reports will be submitted to the FDA.

Telephoned IND Safety Reports

In addition to the subsequent submission of a written IND Safety Report (i.e., completed FDA Form 3500A), the Investigator-Sponsor will notify the responsible review division of the FDA by telephone or facsimile transmission of any observed or volunteered AE that is a.) associated with the use of the investigational drug or study treatment(s); b.) fatal or life-threatening; and c.) unexpected.

The telephone or facsimile transmission of applicable IND Safety Reports will be made as soon as possible but in no event later than 7 calendar days after the Investigator-Sponsor's initial receipt of the respective human AE information.

8.3.2 Reporting Adverse Events to the Responsible IRBs

In accordance with applicable IRB policies of the Veterans Affairs Medical Center Research and Development Committee, University of California, San Francisco Committee on Human Research, and the U.S. Army Medical Research and Materiel Command Human Research Protection Office (USAMRMC HRPO), the Investigator-Sponsor will report, to the IRBs, any observed or volunteered AE that is determined to be associated with the investigational drug or study treatment(s), serious, and unexpected. AE reports will be submitted to the IRBs in accordance with the respective IRB procedures.

Applicable AEs will be reported to the IRBs as soon as possible and, in no event, later than 10 calendar days following the Investigator-Sponsor's receipt of the respective information. Follow-up information to reported AEs will be submitted to the IRB as soon as the relevant information is available. If the results of the Investigator-Sponsor's follow-up investigation show that an AE that was initially determined to not require reporting to the IRB does, in fact, meet the requirements for reporting, the Investigator-Sponsor will report the AE to the IRB as soon as possible, but in no event later than 10 calendar days after the determination was made.

In accordance with the USAMRMC HRPO requirements, unanticipated problems involving risk to volunteers or others, serious adverse events related to participation in the study and all subject deaths related to participation in the study should be promptly reported by phone (310-619-2165), by e-mail (hsrrb@amedd.army.mil), or by facsimile (301-619-7803) to the USAMRMC HRPO. A complete written report should follow the initial notification. In addition to the methods above, the complete report can be sent to the USAMRMC, ATTN: MCMR-ZB-P, 504 Scott Street, Fort Detrick, Maryland, 21702-5012.

The Medical Monitor is required to review all unanticipated problems involving risk to subjects or others, serious adverse events and all subject deaths associated with the protocol and provide an unbiased written report of the event to the USAMRMC HRPO. At a minimum, the Medical Monitor should comment on the outcomes of the event or problem and in the case of a serious adverse event or death, comment on the relationship to participation in the study. The Medical Monitor should also indicate whether he/she concurs with the details of the report provided by the Investigator-Sponsor. Reports for events determined by either the Investigator-Sponsor or Medical Monitor to be possibly or definitely related to participation and reports of events resulting in death will be promptly forwarded to the HRPO.

8.3.3 Reporting of Adverse Events to Actelion Pharmaceuticals

Copies of all periodic safety reports (including draft versions for review) to be submitted to the FDA will be provided to Actelion at least 10 days prior to their submission to the FDA. Copies of any MedWatch forms submitted to the FDA will be provided to Actelion immediately upon submission to the FDA.

All serious adverse events, regardless of causality and expectedness, will be reported to Actelion within 24 hours of the Investigator-Sponsor's knowledge of the event.

8.3.4 Withdrawal of Subjects Due to Adverse Events

Withdrawal of subjects due to an AE can take place at any time during the study at the discretion of the Investigator-Sponsor. Subjects may also choose to discontinue participation at any time.

9. STATISTICAL METHODS/DATA ANALYSIS

9.1 Study Endpoints

9.1.1 Analysis of Primary Endpoints

It is hypothesized that subjects receiving zolpidem 10mg will show greater impairment in neurocognitive performance and objective measures of sleepiness compared to subjects

receiving placebo, almorexant 100mg, or almorexant 200mg. This hypothesis will be tested by comparing groups on post-medication performance tests using pre-medication test scores as covariates. When multiple administrations of a performance test are given either pre-or post-medication, mixed effects models will be used, with the group by time (pre- or post-medication) interaction effect serving as the test of the hypothesis. When a test is administered only once pre- and post-medication, the statistical test will be a one-way ANCOVA comparing mean scores on the four groups, with the pre-medication test score serving as the covariate. Covariates in all models will include total sleep time measured by PSG on the night before testing and average sleep duration measured by actigraphy. Planned comparisons will be conducted to compare the zolpidem 10mg group with placebo, almorexant 100mg, and almorexant 200mg separately. Post-hoc comparisons will be made to compare placebo vs. almorexant 100mg, placebo vs. almorexant 200mg, and almorexant 100mg vs. almorexant 200mg. For post-hoc comparisons, p-value adjustments will be made using a re-sampling procedure as implemented in the SAS "simulate" adjustment option.

Two-tailed significance tests will be conducted at the p = .05 level. P-value adjustments will be made for multiple endpoint variables within each domain of neurocognitive functioning (verbal memory, attention/working memory, motor skills, executive function, and psychomotor vigilance) and objective sleepiness (sleep onset latency and low frequency EEG power in the MWT). The p-value adjustments will be made using a step-down, re-sampling based procedure (38, 39) which takes into account the correlational structure among the multiple variables. Primary analyses will be intent-to-treat analyses based on all participants randomized, regardless of dropout or missing data status. Dropout rate will itself be analyzed as a secondary outcome variable. Missing data will be carefully characterized, and multiple imputation will be used where necessary. The exact form of each mixed model, for example the correlational structure of repeated measures and whether heterogeneous group variances are included, will be made on the basis of best fit according to the Bayesian Information Criterion (BIC) before any hypothesis testing is conducted. Assumptions of the models (e.g., normal distributions of errors and absence of outliers) will be assessed, and any necessary remedies, such as data transformation or the use of robust standard errors, will be implemented before hypothesis tests are conducted.

Any deviations from the statistical plan will be described in the study manuscript.

9.1.2 Analysis of Secondary Endpoints

Secondary endpoints include sleep latency on the MWT measured beyond the presumed drug activity period at 270 and 390 minutes post-dose (i.e., the "hangover effect"), and subjective sleepiness measured by the Stanford Sleepiness Scale. Secondary analyses will be conducted in a parallel fashion to the primary analyses, but with re-sampling based multiple comparison procedures for all significance tests.

9.2 Sample Size Determination

Enrollment is estimated to include up to 216 subjects to obtain 200 evaluable subjects. An equal number of subjects (up to 54) will be randomly assigned to each dosing group (almorexant 100 mg, almorexant 200 mg, zolpidem 10 mg, placebo). Randomization will be stratified on the basis of gender and caffeine use. With a power of 0.80 and an alpha of 0.05, the planned sample size will allow for the detection of effect sizes (Cohens' f) of approximately 0.29. It is estimated that the effect of zolpidem 10 mg versus placebo on the cognitive performance measures will range from f = 0.34 to f = 0.80, based on prior findings. Given the hypothesis that both doses of almorexant will be associated with significantly less impairment than zolpidem 10mg, it is possible that a range of effect sizes might be found with almorexant. If almorexant is absolutely no different than placebo, the study will be slightly overpowered to demonstrate its superiority over zolpidem. However, if almorexant has a more subtle impairment effect on cognition, intermediate between that seen with zolpidem 10 mg and placebo, it might become necessary to be able to detect somewhat smaller effects. According to guidelines suggested by Cohen (33), an effect size of f = .14 is considered "small" and f = .39 is considered "medium." Thus, the proposed study is well powered to test its main hypotheses.

9.3 Definition of Analysis Populations

Primary analyses will be intent-to-treat analyses based on all participants randomized, regardless of dropout or missing data status. If there are a substantial number of participant dropouts, separate analyses on completers only will be conducted as a sensitivity analysis, but hypothesis tests will be based on the intent-to-treat sample. No subgroup analyses are planned.

9.4 Safety Analysis

Dosing groups will be compared on each symptom included as part of the Symptom Checklist using Fisher's exact tests or Chi-Square approximations, depending on the frequency of each symptom. No p-value adjustments will be made.

10. QUALITY CONTROL (QC) AND QUALITY ASSURANCE

The study will be carried out according to requirements of the FDA and all other applicable agencies in addition to ICH accepted standards of GCP. All study-specific procedures will be performed according to approved written Standard Operating Procedures. Study monitors will be responsible for ensuring adherence to FDA and ICH guidelines. Study Monitors for this study will be provided by an external contract monitoring group. Regular monitoring of study data and files at the clinical study sites will be performed as defined in the study-specific monitoring plan. Additionally, an authorized representative from the Investigator-Sponsor study team will perform an annual review of study files and training files to ensure adherence to GCP guidelines and study-specific standard operating procedures. Data collected during the study will be subjected to a thorough quality control review by the lead data managers prior to the statistical analysis. Specific requirements related to the data management QC of the study

data will be detailed in the Data Management Plan. AE data will be reviewed on an ongoing basis with the Investigator-Sponsor.

11. DATA HANDLING, RECORD KEEPING, AND CONFIDENTIALITY

11.1 Data Recording/Case Report Forms (CRFs)

A CRF will be completed for each subject enrolled into the clinical study. The Investigator-Sponsor will review each completed CRF book and will complete the Investigator Statement. Completion of the Investigator Statement CRF confirms the Investigator-Sponsor's responsibility for ensuring that all data and corrections on the CRF are complete, accurate, and authentic.

Source documents will consist of laboratory and medical history records, screening instruments, actigraphy data, sleep diaries, PSG data, neurocognitive assessments, and subjective symptom measures including the Symptom Checklist, the Stanford Sleepiness Scale, and AE and concomitant medication disclosures. All necessary information from the source documents will be recorded on the CRFs. Where appropriate, certain data files will be merged with the study database electronically. Data recorded on the CRFs will be identical to the data recorded on the source documents. Queries will be issued to address all discrepancies noted within the study data. Any changes made to the study data as the result of a resolved query will be documented in the audit trail within the study database. Specific procedures related to the handling of blank, discrepant, or otherwise spurious data will be detailed in the Data Management Plan. When all data have been entered, validated and queries resolved, the database will be locked.

11.2 Record Maintenance and Retention

The Investigator-Sponsor will maintain records in accordance with GCP guidelines and all applicable regulations and policies, to include:

- FDA correspondence related to the IND and clinical protocol, including copies of submitted Safety Reports and Annual Reports
- IRB correspondence (including approval notifications) related to the clinical protocol, including copies of AE reports and annual or interim reports
- Current and past versions of the IRB-approved clinical protocol and corresponding IRB-approved consent form(s) and, if applicable, subject recruitment advertisements
- Signed FDA Form 1572 Statements of Investigator
- Financial disclosure information
- Curriculum vitae for the Investigator-Sponsor and all clinical protocol sub-investigators and study personnel
- Certificates of required training for Investigator-Sponsor, all sub-investigators, and other relevant study team members
- Listing of printed names/signatures of Investigator-Sponsor and listed sub-investigators

- Normal values for laboratory ranges
- Laboratory certification information
- Instructions for on-site preparation and handling of the investigational drug, other study treatments, and study materials
- Standard procedures for decoding and breaking the study blind
- Master randomization list
- Signed informed consent forms
- Completed Case Report Forms, signed and dated by the Investigator-Sponsor
- Source Documents
- Monitoring visit reports
- Copies of Investigator-Sponsor correspondence to sub-investigators, including notifications of safety information
- Subject screening and enrollment logs (a listing of all volunteers who signed informed consent)
- Subject identification code list
- Investigational drug dispensing and accountability records, including documentation of drug disposal
- Final clinical study report

The Investigator-Sponsor will retain the specified records and reports for a minimum of two years after the marketing application is approved for the investigational drug. If a marketing application is not submitted or approved for the investigational drug, records will be retained until 2 years after investigations under the IND have been discontinued and the FDA so notified.

11.3 Confidentiality

Participation in research will involve a loss of privacy, but information about subjects will be handled as confidentially as possible. Medical records will be created at UCSF and SFVAMC because of subjects' participation in this study. Information related to informed consent and screening test results will be included in the medical records, as well as information pertaining to vital signs, adverse events, and concomitant medications assessed during the hospital portion of the study. Therefore, other doctors may become aware of the individual's study participation. Hospital regulations require that all health care providers treat information in medical records confidentially. At the time of consent, subjects will be asked to sign forms to authorize the release of their personal health information for research purposes.

If it is suspected that the subject is in danger of harming him/herself or someone else, or if child abuse or neglect or elder abuse has occurred, appropriate authorities will be notified as required by law. It is also possible that subjects' research records could be subpoenaed by a court.

If information from this study is published or presented at scientific meetings, subjects' names and other personal information will not be used.

All study data will all be coded with a code number unique to the study. Only study personnel, with the permission of the Investigator-Sponsor, will have access to the key with the name and ID codes. The subject identification code list will be stored electronically in a password-protected, restricted access folder on a secured study server in order to maintain confidentiality. The only individuals receiving access to the code list will be the team member responsible for maintaining the list and a back-up.

The clinical interviews performed at screening will be audio recorded and will be used only by research personnel in order to calibrate the clinicians' ratings on the standardized interview format. The neurocognitive assessments performed on Day 10 will also be recorded for QC and calibration purposes. All recordings will be labeled with a unique code number and retained in a secure location (digital recordings will be encryped, passcode protected, and stored and accessed via the secure VA server). Recordings will be retained until the conclusion of the study; at that point, they will be erased. Subjects will be informed that their screening clinical interviews will be audio recorded for the purpose of allowing the research team to ensure consistency across all clinical interviews. They will be informed that the recordings will be maintained under secure conditions at all times and identified only by the unique Subject ID number. Subjects will also be informed that the recordings will be deleted after the conclusion of the study.

The Maintenance of Wakefulness Tests performed on Day 10 will be video recorded and will be used only by research personnel for the purpose of confirming subjects' ability to remain awake during the testing process. The recordings will be labeled with a unique code number and retained in a secure location (digital recordings will be encryped, passcode protected, and stored and accessed via the secure VA server). Recordings will be retained until the conclusion of the study; at that point, they will be erased. Subjects will be informed that their Maintenance of Wakefulness Tests on Day 10 will be video recorded for the purpose of allowing the research team to confirm their ability to remain awake during testing. They will be informed that the recordings will be maintained under secure conditions at all times and identified only by the unique Subject ID number. Subjects will also be informed that the recordings will be deleted after the conclusion of the study.

Organizations that may look at and/or copy subjects' medical records for research, quality assurance, and data analysis include representatives from the following:

- UCSF CHR
- FDA
- USAMRMC
- Actelion Pharmaceuticals, Ltd.

12. ETHICS

12.1 Institutional Review Board (IRB) approval

Prior to initiating the study, the Investigator-Sponsor will obtain approval in writing from all required IRBs. Specifically, approval must be obtained from the UCSF Committee on Human Research, the Veterans Affairs Research and Development Committee, and the U.S. Army Medical Research and Materiel Command Office of Research Protections Human Research Protection Office.

Any amendments to the protocol or changes to the informed consent document must be approved by all IRBs prior to the implementation of those changes. The only circumstance in which a modification to the current IRB-approved clinical protocol/consent form(s) may be initiated in the absence of prospective IRB approval is to eliminate an apparent immediate hazard to the research subject(s). In such circumstances, the Investigator-Sponsor will promptly notify the IRBs of the modification.

The IRBs will be promptly notified of any deviation to the protocol that may have an effect on the safety of the subjects and the integrity of the study. This notification will occur as soon as the deviation is identified. All deviations will also be reported in the continuing review report and final study report.

A copy of the approved continuing review report and the local IRB approval notification will be submitted to the USAMRMC ORP HRPO as soon as these documents become available. A copy of the approved final study report and local IRB approval notification will be submitted to the USAMRMC ORP HRPO as soon as these documents become available.

In the event that the IRB requires, as a condition of approval, substantial changes to a clinical protocol submitted under an FDA-accepted IND application, or in the event of an Investigator-Sponsor's decision to modify the previously accepted clinical protocol, the Investigator-Sponsor will submit a protocol amendment (prior to the implementation of the changes) to the IND describing any change to the protocol that would significantly affect the safety of subjects, the scope of the investigation, or the scientific quality of the study.

Records of IRB approval and other related correspondence will be maintained in the regulatory files for the study and will be subject to periodic audits and reviews by study monitors. Periodic status reports will be submitted to the IRB as required, and AEs/serious AEs will be reported to each IRB per their specific reporting requirements.

12.2 Ethical and Scientific Conduct of the Clinical Study

The clinical study will be conducted in accordance with the current IRB-approved clinical protocol, ICH Guidelines on GCP, and relevant policies, requirements, and regulations of the FDA, UCSF CHR, the VA R&D Committee, the USAMRMC ORP HRPO, and all other applicable state and federal agencies. All procedures described in

this protocol will be performed according to approved written SOPs unless otherwise stated.

12.3 Subject Informed Consent

The Investigator-Sponsor will make certain that an appropriate informed consent process is in place to ensure that potential research subjects are fully informed about the nature and objectives of the clinical study, the potential risks and benefits of study participation, and their rights as research subjects. The Investigator-Sponsor, or a staff member designated by the Investigator-Sponsor, will obtain the written, signed informed consent of each subject prior to performing any study-specific procedures. The date and time that the subject signs the informed consent form and a narrative of the issues discussed during the informed consent process will be documented in the subject's case history. The Investigator-Sponsor will retain the original copy of the signed informed consent form and a copy will be provided to the subject.

The Investigator-Sponsor will make certain that appropriate processes and procedures are in place to ensure that ongoing questions and concerns of enrolled subjects are adequately addressed and that the subjects are informed of any new information that may affect their decision to continue participation in the clinical study. In the event of substantial changes to the clinical study or the risk-to-benefit ratio of study participation, the Investigator-Sponsor will obtain the informed consent of enrolled subjects for continued participation in the clinical study

13. EARLY DISCONTINUATION CRITERIA

A subject may withdraw or be withdrawn from the study for the following reasons:

1.) Subject withdrew consent
2.) Investigator judgment
3.) Protocol violation(s)
4.) Non-compliance
5.) Adverse Event
6.) Pregnancy
7.) Other

If subjects withdraw consent prior to admission to the CCRC, they will be asked to return to the SFDVAMC for an early discontinuation visit which will entail an assessment of AEs and concomitant medications, a debriefing, and the return of study-related equipment.

If it becomes necessary to stop parts or all of the clinical study for the safety of the subjects, Actelion, the IRBs, and the FDA will be notified promptly of the discontinuation of the entire clinical study. Respective protocol modifications will be submitted prospectively to the IRB and to the FDA for discontinuation of parts of the clinical study. All sub-investigators will be notified of any necessary discontinuations.

Subjects participating in the study at the time of the discontinuation of a portion or all of the study will be promptly notified and advised of the impact of the discontinuation upon their study schedules. If a portion of the study is discontinued, subjects will be provided with revised informed consent documentation which will need to be signed prior to their continued enrollment in the study.

14. RISKS AND BENEFITS

Study-related risks and associated measures to minimize the risks are listed below:

Study Drug Related Side Effects

Some subjects might experience side effects associated with the study drugs. The list of possible side effects presented below is based on side effects that have been observed in clinical trials involving Almorexant and Zolpidem. Participants in these clinical studies took many different dosages of these drugs ranging from 1mg to 1000mg. Subjects will be told to discuss any side effects with study personnel as they occur. The nursing staff at the CCRC and study personnel will also closely monitor subjects on the day of dosing with study drug. All subjects will have a liver function test performed within 5 – 14 days of dosing with study drug.

Risks and side effects related to taking Almorexant include those which are:

Likely (occurring in greater than 20% of people)

- Drowsiness

Less Likely (occurring in less than or equal to 20% of people)

- Fatigue
- Headache
- Dizziness
- Nausea
- Liver Enzyme Elevations (mainly with administration for longer than two weeks of daily almorexant 100mg and 200mg)

Rare but Serious

- Heart rate abnormality (less than 1%)
- Convulsions (less than 1%)

Risks and side effects related to taking Zolpidem include those which are:

Less Likely (occurring in less than or equal to 20% of people)

- Dizziness
- Drowsiness
- Headache
- Diarrhea
- Fatigue

Rare but Serious

- Heart rate abnormality (less than 1%)
- Severe allergic reaction (less than 1%)

Also, in rare cases (.1% - 1%), people taking Zolpidem have reported engaging in unusual behaviors (e.g., driving, preparing and eating food, or making phone calls) while not being fully awake after taking Zolpidem, with no later memory of the events. However, these events occurred when people went to sleep after taking Zolpidem. During this study, subjects will remain awake and in a controlled, monitored setting after being given the study drug, so unusual behaviors are unlikely to occur.

Lastly, data has shown that small amounts of Zolpidem present in the blood up to 8 hours after taking the drug can harm performance in tasks involving full alertness, such as driving. This risk is most applicable to women, but could also apply to men. Because of this, the FDA has lowered the currently recommended dose of Zolpidem for women from 10mg to 5mg. If a subject is randomly assigned to take Zolpidem in this study, he/she will be given 10mg, which is twice the recommended dose for women. However, subjects will be given study drug at 3pm on Day 10 and will not be discharged until 8am the next day (17 hours after taking the sleep aid), so driving impairments are unlikely to occur.

Blood Drawing (Venipuncture)
The risks of drawing blood include temporary discomfort from the needle stick, bruising, and rarely, infection. The amount of blood collected to determine eligibility is approximately 20 ccs or 4 teaspoons. Only a qualified phlebotomist will draw blood following standard SFVAMC lab procedures.

Clinical Interview & Questionnaires
The interview and questionnaires may be distressing to some participants. Subjects will be told that they are free to decline to answer any questions or to stop the interviews at any time.The interviewer will be available to immediately assist with any problems that arise in the interview and will make a referral if required.

Audio Recording – Clinical Interview and Neurocognitive Tests
The clinical interviews and some of the neurocognitive tests will be audio taped. The audio taping may make subjects somewhat more uncomfortable than they would be without the taping. Research personnel will use the recordings in order to ensure that study staff are administering and scoring the tests correctly and in the same way. The audio recordings will be maintained under secured conditions (i.e., the recordings will be encrypted, protected with a pass code, and stored and accessed via a secure server), identified only by a unique ID number, and retained until the conclusion of the study, at which point they will be erased/deleted.

Actigraphy
There is no risk of injury from wearing the actigraph. Subjects might find it annoying to have to wear the actigraph 24 hours per day during the 10 day study. Subjects will be told that they can discuss any difficulties with this procedure with study personnel at any time. Subjects will also be able to decline to participate in this procedure at any time.

Polysomnography
There is no risk of injury from any of the recording devices, but subjects might experience slight discomfort from the attached electrodes and tape. High quality hypoallergenic materials will be used to minimize this risk.

Video Recording – Maintenance of Wakefulness
The Maintenance of Wakefulness Tests that will be conducted on Study Day 10 will be videotaped. The video recording may make subjects somewhat more uncomfortable than they would be without the taping. These recordings will only be reviewed by research staff and our consultants for the purpose of confirming subjects' ability to remain awake during the testing. The recordings will be identified by a unique ID number and will be stored under secure conditions (i.e., they will be encrypted, protected with a pass code and stored on a secure server). The recordings will be retained until the conclusion of the study, at which point they will be destroyed.

Maintenance of Wakefulness Tests
There is no risk of injury from taking this test, but subjects might find it annoying or difficult to remain awake while sitting quietly in a comfortable position. Subjects might also become bored while sitting still for the 20 minute duration of the test. Subjects will be able to stop the procedure at any time if they become uncomfortable.

Neurocognitive Assessment Battery
There is no risk of injury from completing the neurocognitive assessment battery, but subjects might become bored, frustrated, or find it difficult to concentrate as you take these tests throughout the day of testing. Subjects will be able to stop the procedures at any time if they become uncomfortable.

Sleepiness
There is a 3 out of 4 chance that subjects will take a sleep aid on Study Day 10 while at the hospital. Therefore, subjects might become sleepy during the study testing procedures, and the study staff will require subjects to remain awake. This might be difficult or frustrating for subjects.

Reproductive Risks
Subjects should not become pregnant or father a baby while participating in this study because the potential effects of the study drugs on an unborn baby are not known at this time. Women should not breastfeed a baby while on this study. Study staff will educate subjects regarding the importance of using appropriate birth control throughout the study.

Unknown Risks
The experimental drugs used in this study may have side effects or discomforts that no one knows about yet. Subjects will be told to discuss any side effects with study personnel as they occur. The nursing staff at the CCRC and study personnel will also closely monitor subjects on the day of dosing with study drug.Subjects will not experience any direct benefits by participating in the study. However, the study is contributing to medical knowledge related to the cognitive effects of sleep aids. Results

could have implications for personnel of the military and/or other professions who have an occupational risk of poor sleep.

15. STUDY PERSONNEL

15.1 Investigator-Sponsor

The Investigator-Sponsor will assume overall scientific and administrative leadership for the study. He will be responsible for supervising the study team with regards to the recruitment, diagnostic assessment, and enrollment of subjects and the coordination of all study procedures.

The Investigator-Sponsor will have overall responsibility for the standardization of data collection, data quality control, data analysis, and interpretation. He will have overall responsibility for subject safety, rights, and welfare. He will be an active participant in the preparation of abstracts and manuscripts and will assure the dissemination of study findings in the professional and scientific communities.

15.2 Medical Monitor

The Medical Monitor may be asked to discuss research progress with the Investigator-Sponsor, consult on individual cases, or evaluate adverse event reports for the safety and protection of the subjects. The Medical Monitor shall promptly report discrepancies or problems to the IRB and the HRPO, and he will have the authority to stop a research study in progress, remove individual subjects from a study, and take whatever steps are necessary to protect the safety and well-being of research volunteers until the IRB can assess the Medical Monitor's report. At a minimum, the Medical Monitor will provide a written opinion regarding the relationship and outcome of any unanticipated problems related to participation, serious adverse events, and subject deaths.

15.3 Co-Investigators

The Co-Investigators assigned to this study will assist the research team in data collection, data analysis, quality control of study data, data interpretation, and the preparation of reports. They will provide consultation and oversight to the mental health clinicians and will assist with the determination of eligibility.

15.4 Study Coordinator

The study coordinator will be responsible for the day-to-day activities of the study, including but not limited to the following: obtaining informed consent, subject scheduling, eligibility determination, ensuring the completion of safety reports in a timely manner, case report form completion, ensuring that study team members are properly trained on study procedures, providing oversight to the external study monitors, and

providing oversight for data completion, cleaning, analysis, and interpretation. The study coordinator will consult with the project director as necessary for high-level study management and budget oversight.

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Appendix 2: Animal Studies Progress Report

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Award Number:

USAMRAA Grant W81XWH-09-2-0081

TITLE:

EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE

PRINCIPAL INVESTIGATOR:

Thomas S. Kilduff, Ph.D.

CONTRACTING ORGANIZATION:

SRI International
333 Ravenswood Avenue
Menlo Park, CA 94025-3493

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
During Year 5, we continued tests of the hypothesis that disfacilitation of wake-promoting systems by the hypocretin (Hcrt) receptor antagonist almorexant (ALM) results in less functional impairment than the inhibition of neural activity produced by the benzodiazepine receptor agonist zolpidem (ZOL). One paper was published (Morairty et al. 2014), another has been accepted for publication (Dittrich et al., in press) and a third is in resubmission (Vazquez-DeRose et al., submitted). Data collection for Aims 2c and 3b.2 have been completed and data analysis ongoing; manuscripts will be written and submitted during Year 6. Data collection and analysis of Aim 3a is nearing completion; an abstract summarizing this work has been submitted for presentation at the 2014 Society for Neuroscience meeting. Data collection for Aims 3b.3, 4c and 6a have been initiated. The overall results obtained to date are consistent with the hypothesis that the hypocretin/orexin antagonist ALM produces less functional impairment than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems.

15. SUBJECT TERMS
Sleep, performance, drug, neurotransmitter, hypocretin, orexin, benzodiazepine, zolpidem, neurochemistry, microdialysis

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PROGRESS REPORT

"Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance"

USAMRAA Grant W81XWH-09-2-0081

DR080789P1

Year 5: 8/1/13 to 7/31/14

Thomas S. Kilduff, Ph.D., Principal Investigator

INTRODUCTION

Almorexant (ALM) is a hypocretin/orexin (Hcrt) receptor antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The overall hypothesis that underlies this research is that ALM produces less functional impairment than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. Whereas the human study component (W81XWH-09-2-0080; Thomas Neylan, M.D., Principal Investigator) will establish whether ALM is superior to ZOL in neurocognitive tests, the animal studies (W81XWH-09-2-0081; Thomas Kilduff, Ph.D., Principal Investigator) will compare the neural circuitry that underlies the activity of these compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

BODY

Task 2. *Test the hypothesis that rodents receiving ZOL will show greater neurocognitive impairment than those receiving ALM or PBO.*

2a. Assessment of Almorexant effects on spatial reference memory in rats.
Status: Data collection and analysis COMPLETED; paper published in January, 2014.

2b. Assessment of Almorexant effects on spatial working memory in rats:
Status: Data collection and analysis COMPLETED; paper published in January, 2014.

2c. Assessment of Almorexant effects on psychomotor vigilance in rats
Status: Data collection completed; analysis ongoing (see below).

2d. Synthesis of ALM (months 1-4).
Status: COMPLETED

Progress – Task 2a and 2b: Tasks 2a and 2b have been completed and an article entitled "The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats" was published in *Frontiers in Neuroscience* in January, 2014.

Progress – Task 2c: The studies assessing the effects of ALM in the rodent psychomotor vigilance (rPVT) have been completed. Our results are described below.

Methods: The general protocol for the rPVT is as follows (**Figure 1**). Rats were motivated to perform the operant rPVT task for water reinforcements by having water unavailable to them for 23 h prior to all operant training and testing. Rats were gradually acclimated to the water restriction schedule over several days by reducing the amount of time

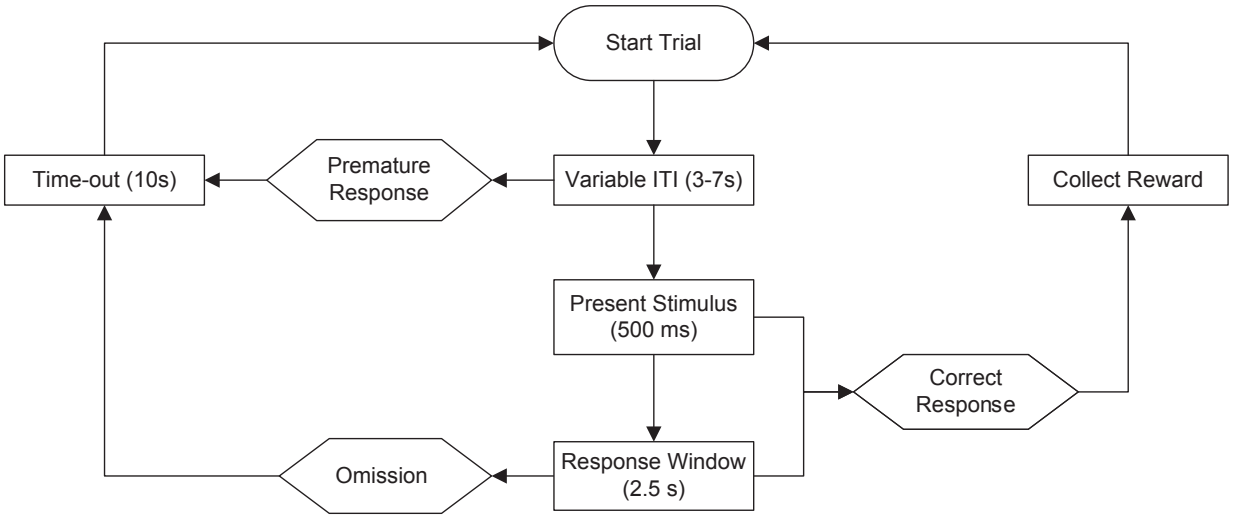

Figure 1. Flowchart of the rodent Psychomotor Vigilance Task. During the task, the inter-trial interval (ITI) varied from 3 to 7 s in 1 s increments in a quasi-random fashion (equal density of intervals throughout session). Responses during the ITI (a 'premature response') or failing to respond within 3 s of the stimulus presentation (an omission) were treated as errors and resulted in a 10 s 'time-out' (housing light extinguished and absence of trials). At the end of the 10 s time-out, the house light was re-illuminated and a new ITI started.

each day that water was available in the home cage. rPVT training took 3 mo to complete. Following this 3 mo training period, rats that did not meet criteria (> 100 correct responses per test session) were removed from the study. rPVT testing consisted of a stimulus light on for a duration of 0.5 s followed by a 3 s response period. The intertrial interval varied between 3-7 s. Errors resulted in a 10 s "time out" period during which the dim house lights were turned off. Test measures were the following:

- Correct responses (**CR**): Responding during stimulus presentation or within the response window.
- Omission (**OM**): Failure to respond within the 3 s window of opportunity.
- Premature errors (**PE**): Responding during the inter-trial interval.
- Response latencies (**RL**): Time from stimulus onset to a correct response.
- Numbers of trials: Total number of trials per session.
- Number of responses: Number of entries in the reward trough (data not shown).
- Lapses: Trials in which response latencies were >2x the average basal response latency for each rat.

Results: Seventeen rats were implanted with telemetry for devices for EEG recordings. Of these, 4 rats did not meet criteria following 3 mo of training and were removed from the study. We anticipated that up to a third of the rats might not meet criteria, so these results were expected. In addition, 2 rats had transmitter malfunctions prior to completion of the study and could not be included in our results. Therefore, 11 rats completed the rPVT study.

When the testing was about to begin, rats were acclimated to the dosing procedure by administration of 1 ml of VEH (p.o.). However, when we examined the performance following this dose of VEH, we found a significant decline in all rPVT measure. Therefore, we reformulated the VEH solution using a base of physiological saline rather than just H_2O. This

reformulation was effective at keeping the rats' performance in the rPVT above minimum criteria following dosing with VEH.

When the experiments were initiated, it became clear very early on that there were significant deficits in performance following ZOL at 100 mg/kg, p.o. Some rats had very few responses to the stimulus following ZOL. In addition, ALM-treated rats showed a noticeable deficit compared to VEH. Therefore, we added 2 additional conditions, ALM and ZOL at 30 mg/kg (p.o.). These additional concentrations of ALM and ZOL have been shown to be sleep-promoting but at more moderate levels compared to 100 mg/kg doses.

While performance in the rPVT declined following ALM and ZOL at both concentrations, the magnitude of the decline was significantly greater following ZOL (**Figure 2**). All rPVT performance measures decreased significantly following ZOL administration. Following ZOL, CR and the number of trials decreased while OM, response latencies and lapses increased. Interestingly, the number of PE decreased; following ZOL, rats were simply engaging less in the task. While ALM showed a decrease in sustained attention (decreased CR and the number of trials, increased OM and lapses), no impairment was seen in RL or PE. Further, the effects on CR, OM, the number of trials and lapses were greater following ZOL than ALM.

To investigate deeper into rPVT performance, we determined the density distributions for response latencies following all conditions (**Figure 3**). For both VEH and ALM, most responses occurred in less than 0.5 s. and the density distribution patterns of the VEH and ALM were similar. Following ZOL, however, the density distribution showed a much broader distribution across the response period. These data show that rats performed equally as well following ALM as following VEH, while responses often occurred more slowly in the presence of ZOL.

Changes in rPVT performance could not be attributed to prior sleep history. As can be seen in **Figure 4**, rats slept equivalent amounts for the hour prior to testing following ALM and ZOL. However, while the EEG power spectra during NREM sleep following VEH and ALM were indistinguishable (**Figure 5**), ZOL was followed by very large changes across the entire NREM EEG power spectrum (**Figure 6**). While the full meaning of such changes in the EEG power spectrum is yet to be understood, these data support the hypothesis that ALM produces physiological sleep while ZOL produces generalized CNS inhibition that results in a pharmacological, rather than physiological, sleep state.

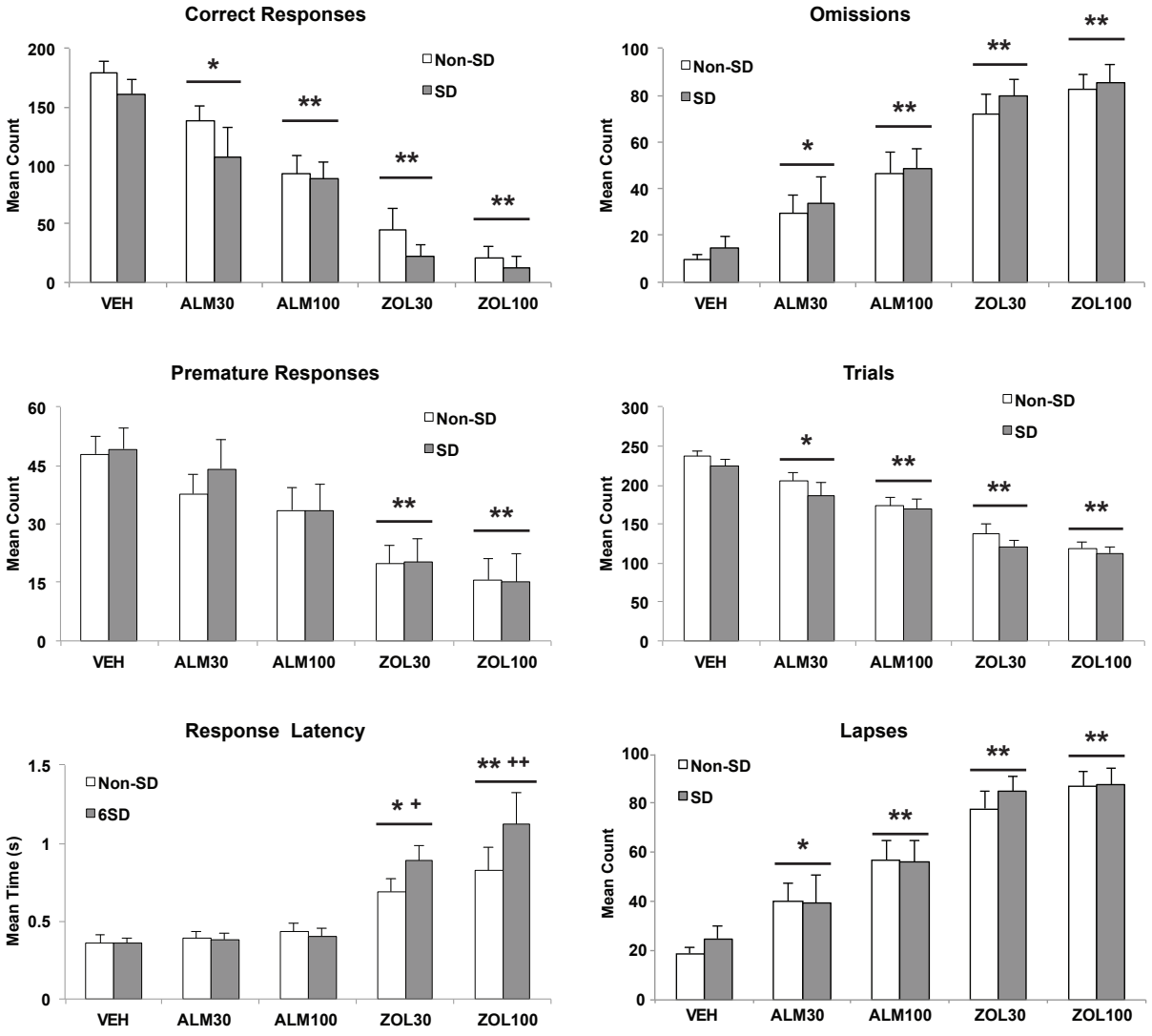

Figure 2. rPVT outcome measures. rPVT performance decreased significantly across all measures following ZOL administration. While ALM showed a decrease in sustained attention (decreased CR, increased OM and lapses), no impairment was seen in RL. Data shown as group mean ± SEM (n = 9–10). Multiple comparisons vs. control group (Bonferroni t-test): *= $p < 0.05$ significantly different from vehicle condition. ** = $p < 0.01$ significantly different from vehicle condition. + = $p < 0.05$ significantly different SD condition within drug treatment. ++ = $p < 0.01$ significantly different SD condition within drug treatment.

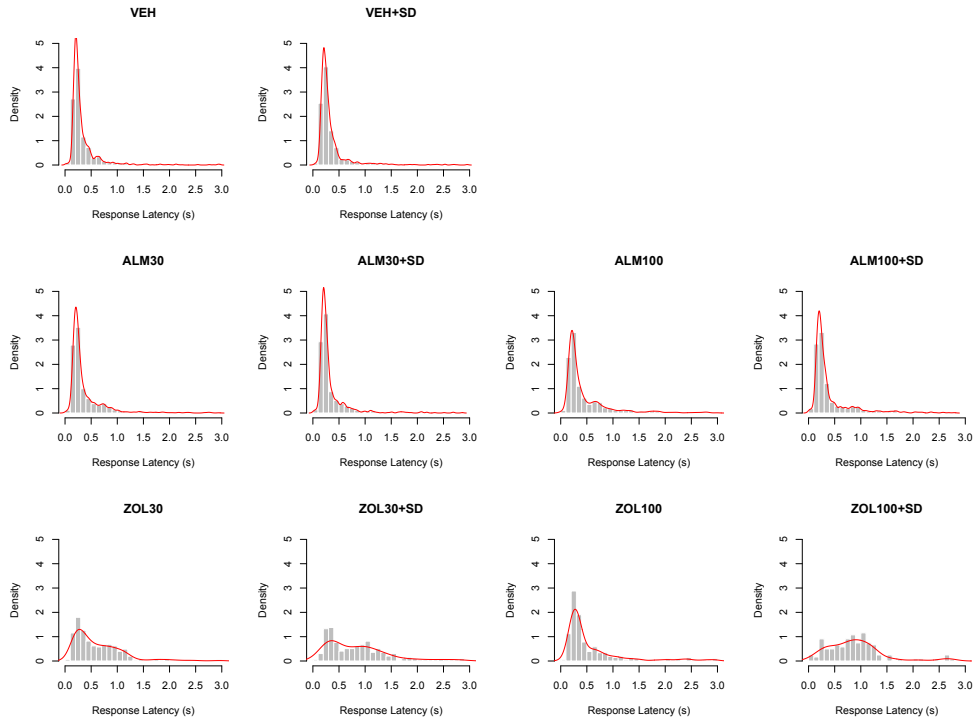

Figure 3. Density distributions for the response latencies in each test condition. RL distributions are similar for VEH and Almorexant (ALM) following either baseline or SD conditions. However, ZOL administration shifted the RL distributions following both baseline and SD conditions.

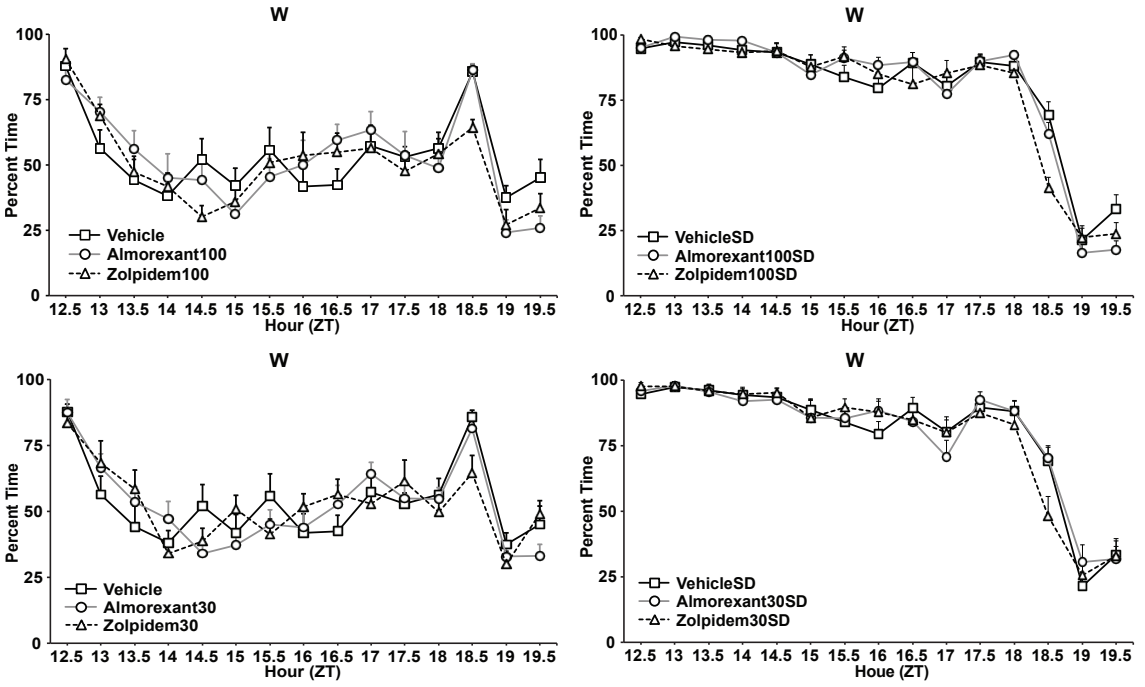

Figure 4. Effects of Almorexant and Zolpidem (each at 100 mg/kg and at 30 mg/kg po) on time spent awake under baseline conditions (left panel) or following 6 h of SD (right panel) during the active phase (lights off). Note that for the 60 min prior to rPVT test, the doses of Almorexant and Zolpidem were equally effective at inducing sleep.

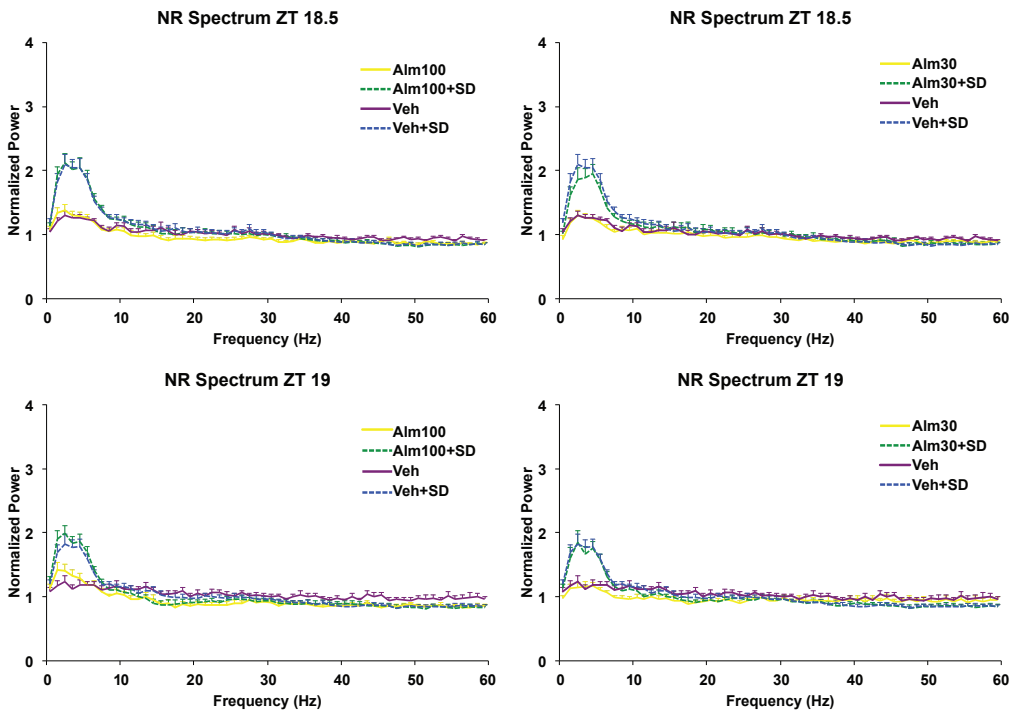

Figure 5. Rats showed no significant differences in EEG spectra during NREM sleep at 30 min (top) or 60 min (bottom) following administration of Almorexant (Alm) when compared to vehicle (Veh).

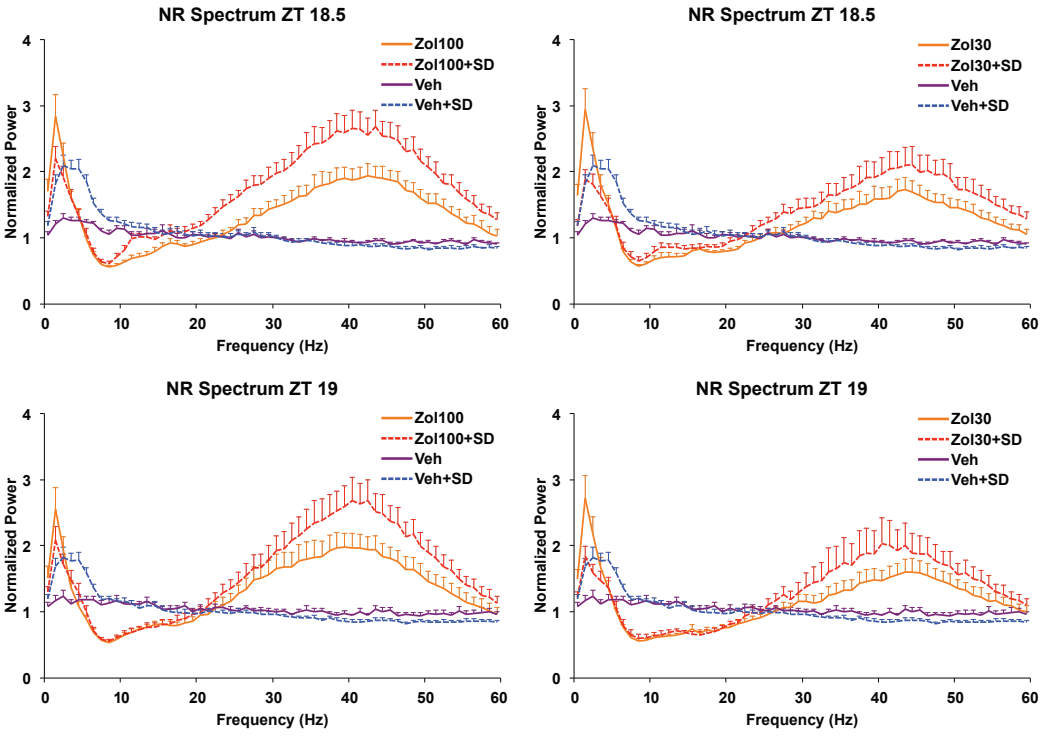

Figure 6. Rats showed significant alterations in EEG spectra during NREM sleep (0-60 Hz at 1 Hz resolution) 30 min (top) and 60 min (bottom) after administration of Zolpidem (Zol; left panel: 100 mg/kg; right panel: 30mg/kg) compared to vehicle (Veh). These changes appear to be dose-dependent and are further potentiated following 6 h of sleep deprivation (SD) during the active phase.

Task 3. *Test the hypothesis that the Hcrt antagonist ALM induces sleep by selectively disfacilitating the activity of the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems whereas the BzRA ZOL causes a generalized inhibition of the brain.*
3a. Double-label immunohistochemistry with Fos and phenotypic markers.
Status: Data collection and analysis ongoing; see below.

3b. Assessment of hypnotic efficacy in saporin-lesioned rats.
3b.1 Status: Basal forebrain lesion study COMPLETED; manuscript in revision for publication in *Brain Structure and Function*.
3b.2 Status: Locus coeruleus lesion study COMPLETED; manuscript to be written.
3b.3 Status: Tuberomammillary nucleus lesion study ongoing; see below.

Progress – Task 3a: We proposed to determine whether ALM and / or ZOL disrupt activation of several wake-promoting neuronal populations during forced wakefulness and undisturbed conditions using Fos as a marker for neuronal activation. Task 3a is nearly complete as all data collection and most histological stains have been completed, but some analysis remains. Completion of Task 3a was delayed because data needed to be collected from 12 additional rats this year, bringing the total utilized to N=88 for this study.

Methods: Rats were given 1 mL (p.o.) ALM (100mg/kg), ZOL (100mg/kg), or VEH at their mid-active phase (ZT18). Half of the animals were left undisturbed for 1.5h after dosing, while the other half of the rats were sleep deprived (SD) by gentle handling for 1.5h. Animals were then deeply anesthetized and perfused, and the brains were removed and sectioned on a freezing microtome. Double label immunohistochemistry for Fos and markers for wake-active neurons (histamine (HA), hypocretin (Hcrt), serotonin (5-HT), and acetylcholine (ACh)) was performed using coronal sections of tissue from the appropriate brain region. Additionally, the number of single labeled Fos-positive nuclei in the locus coeruleus (LC) was quantified.

Results: In HA neurons, both VEH- and ALM-treated rats exhibited significantly greater Fos coexpression with adenosine deaminase (ADA) following SD than did ZOL-treated animals (**Figure 7**). This result indicates that activation of HA neurons is unimpaired by ALM whereas ZOL inhibits such activation.

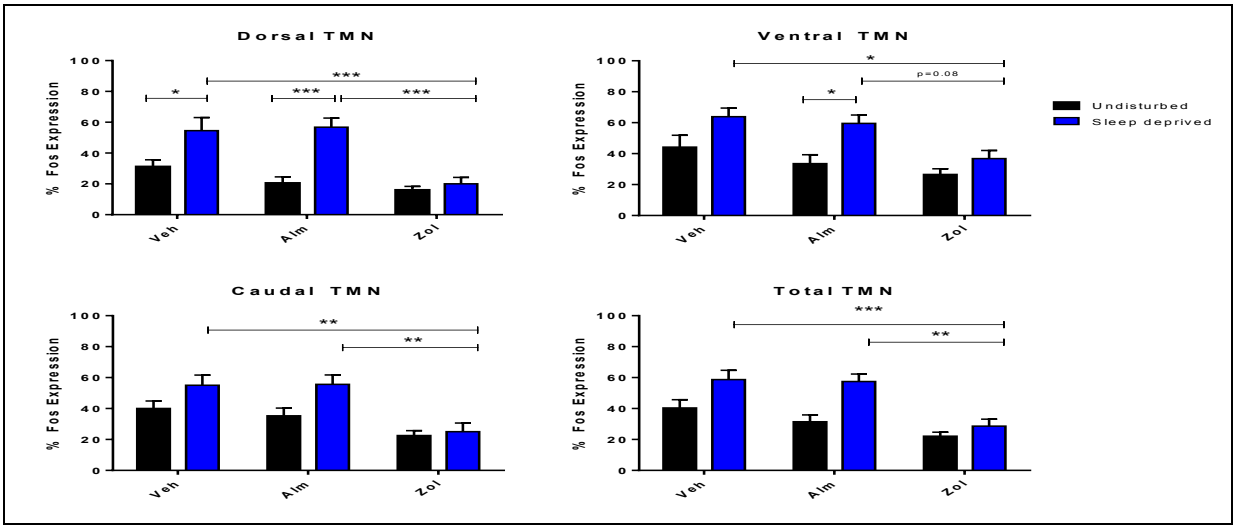

Figure 7. Effect of drug treatment on Fos expression of wake-active HA neurons in the dorsal, ventral and caudal subdivisions of the tuberomammillary nucleus (TMN) of the hypothalamus. Black bars depict group means for undisturbed rats, blue bars for SD rats. *, **, *** = p<.05, 0.01, and 0.001, respectively.

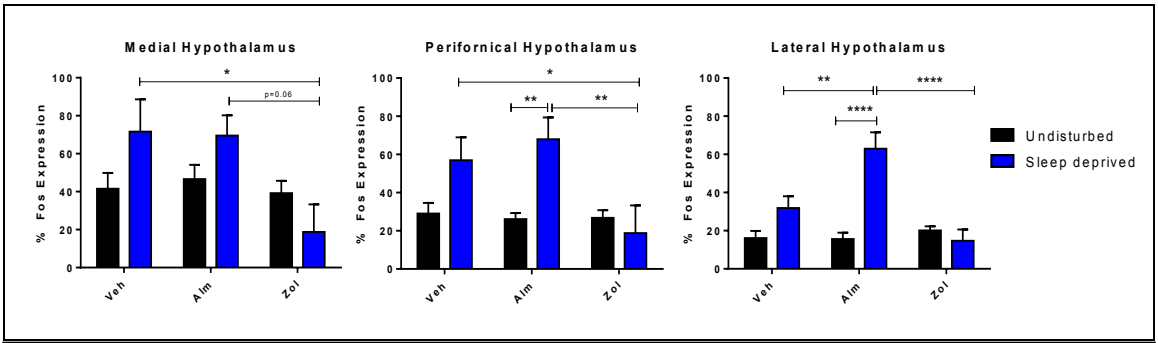

Figure 8. Effect of drug treatment on Fos expression in hypocretin neurons found in three hypothalamic regions. Black bars depict group means for undisturbed rats, blue bars for SD rats. *, **, *** = p<.05, 0.01, and 0.001, respectively.

We also conducted additional processing and analysis of Hcrt double-labeling experiments. The results of this analysis confirm the preliminary results reported last year indicating that a greater proportion of Hcrt neurons express Fos in VEH- and ALM-treated animals after SD than in ZOL animals (**Figure 8).** This indicates that ALM does not impair SD-induced activation of Hcrt neurons, whereas ZOL inhibits activation of this neuronal group.

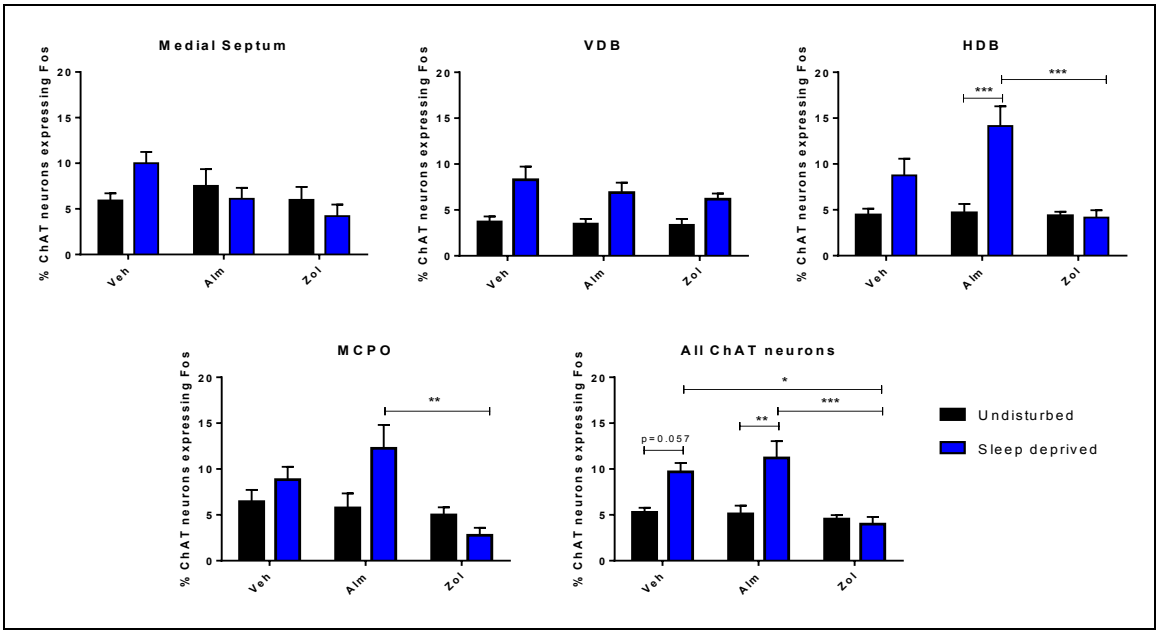

Figure 9. Effect of drug treatment on Fos expression in wake-active cholinergic neurons in four regions of the basal forebrain. Black bars depict group means for undisturbed rats, blue bars for SD rats. *, **, *** = p<.05, 0.01, and 0.001, respectively.

To investigate whether wake-active Ach neurons in the basal forebrain are affected by ALM and ZOL, neurons that express choline acetyltransferase (ChAT), a marker for ACh, were scored for Fos coexpression. In the horizontal diagonal band of Broca (HDB) and magnocellular preoptic nuclei (MCPO), ALM-treated animals expressed significantly greater Fos in ACh neurons than did ZOL-treated animals following SD (**Figure 9**). When ACh neuron counts

across all subregions were consolidated, both VEH- and ALM-treated animals were found to express Fos in a significantly greater proportion of Ach neurons than did ZOL-treated animals. These results indicate that, unlike ZOL, ALM does not inhibit activation of Ach neurons.

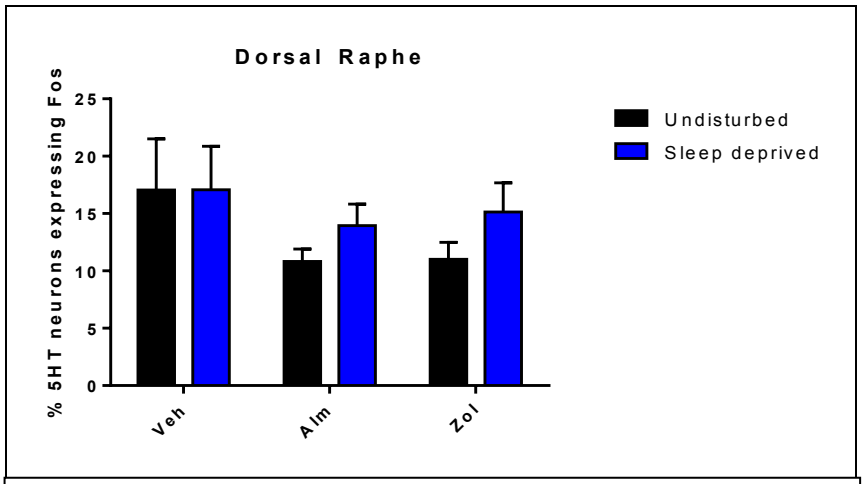

Figure 10. Effect of drug treatment on Fos expression in wake-active 5-HT neurons in the dorsal raphe nucleus. Black bars depict group means for undisturbed rats, blue bars for SD rats.

5-HT expressing neurons in the dorsal raphe nucleus were scored for Fos double labeling. No significant changes in Fos expression were observed between VEH-, ALM-, or ZOL-treated rats either under SD or undisturbed conditions (**Figure 10**), indicating that neither ALM or ZOL affect Fos expression in 5-HT neurons.

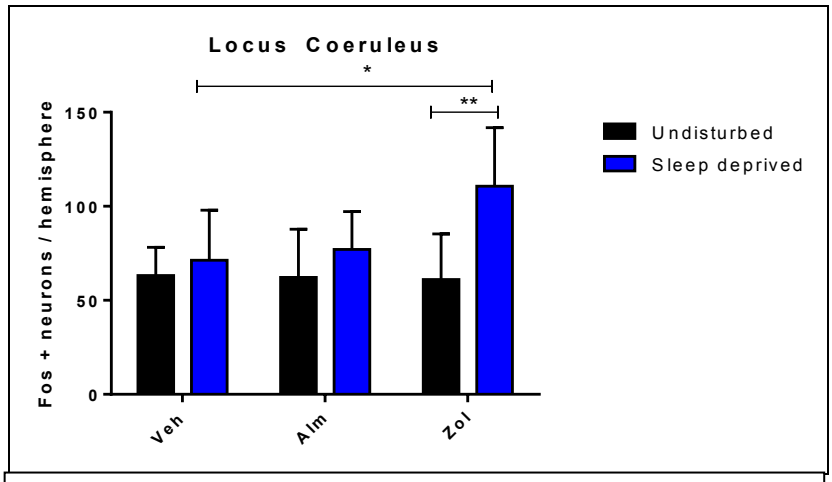

Figure 11. Effect of drug treatment on Fos expression in the locus coeruleus (LC). Black bars depict group means for undisturbed rats, blue bars for SD rats.

The total number of Fos-positive nuclei in a selected region of the locus coeruleus (LC) was counted in order to assess the effect of ALM and ZOL on LC activation. We found that SD did not affect the number of Fos positive nuclei for VEH- or ALM-treated rats, but that ZOL-treated rats exhibited significantly greater Fos expression in this region following SD (**Figure**

11). This is likely a consequence of the increased level of handling required to keep ZOL-treated animals awake during the SD protocol compared to VEH- or ALM-treated rats.

Progress – Task 3b: As described in previous Progress Reports, we have completed two of three lesion studies testing the hypothesis that ALM induces sleep by selectively disfacilitating the activity of subcortical wake-promoting systems. Rats with bilateral lesions of the basal forebrain (BFx) exhibited a decrease in ALM-induced NREM sleep compared to sham-operated rats (Shams), whereas ZOL showed full efficacy in promoting NREM sleep in BFx and Sham rats. The manuscript describing these results is in revision for publication in *Brain Structure and Function*.

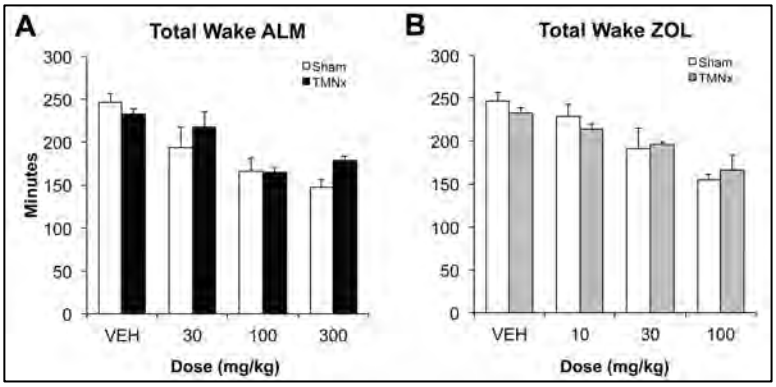

Figure 12. Cumulative wake time for 6 h after dosing with ALM (**A**), ZOL (**B**) or Veh at lights-out (ZT12). Vehicle doses are presented in both graphs. N=2 TMNx, 2 Sham.

Locus coeruleus lesions (LCx) attenuated ALM-induced but not ZOL-induced decreases in NREM sleep latency, and attenuated ALM-induced increases, but not ZOL-induced decreases, in REM sleep compared to Shams. The manuscript describing these results is currently being written.

This year, we initiated the third lesion study and assessed the response to ALM and ZOL following lesions of the histaminergic tuberomammillary neurons (TMNx) of the posterior hypothalamus.

Methods: Adult male rats were injected bilaterally with 250-300 nL of the neurotoxin saporin conjugated to Hcrt2 (Hcrt-SAP; 228ng/μL) using a Hamilton syringe connected to a digital microinjection pump at -4.2mm AP, ± 0.8mm ML from bregma, and -9.3mm from dura. Rats were instrumented for EEG at this time, and following full recovery were administered HPMC vehicle, ALM (30/100/300 mg/kg) or ZOL (10/30/100 mg/kg) p.o. in fully balanced order at lights-out. Sleep EEG was scored for the first 6 h following dosing.

Results: Despite positive results in placing this lesion in pilot studies, only 2 of 8 experimental rats exhibited complete bilateral TMN lesions. Statistical evaluation was precluded due to the low sample size, but TMNx appeared to attenuate the efficacy of ALM 300 mg/kg at reducing wakefulness (**Figure 12A**). By contrast, ZOL exerted more similar reductions in wakefulness in TMNx and Sham rats (**Figure 12B**). While additional rats will be needed to complete the study, these preliminary results are encouraging.

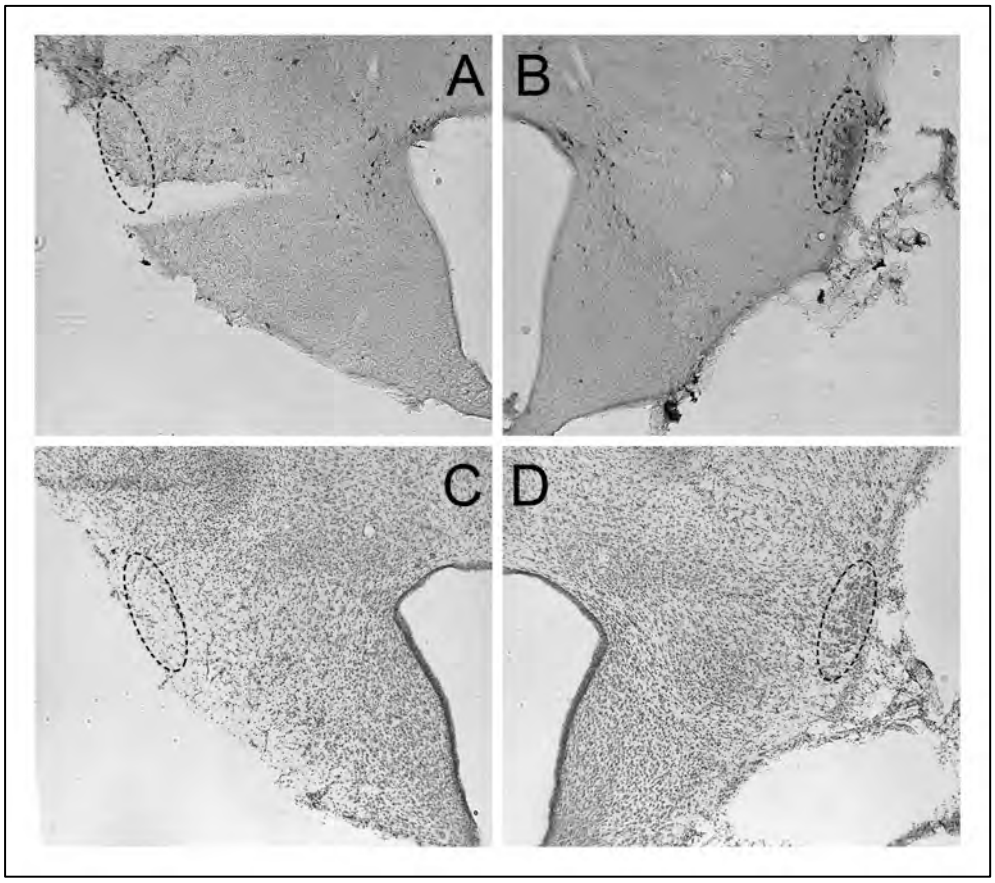

Figure 13. Representative unilateral TMN lesion from a rat injected with 360nL of HCRT-SAP in the left TMN (Panels A &C). Brain sections were immunostained for adenosine deaminase (A-B), with adjacent sections stained for Nissl (C-D). Dotted ovals indicate the location of the TMN; Histamine-positive neurons are evident in the right TMN (B) but not the left (A).

In a second round of pilot studies we refined the lesion placement technique. 8 rats were injected unilaterally with 360nl (N=4) or 540nL (N=4) of Hcrt-SAP (228ng/μL) using calibrated pulled glass micropipettes and a Picospritzer at either 4.2mm (N=4) or 4.35mm (N=4) posterior to bregma (ML and DV coordinates were -0.8mm from bregma and -9.3mm from dura, respectively) and perfused 2 weeks later. 4 of 4 rats injected at -4.35mm AP exhibited a TMN lesion ipsilateral to the injection site (**Figure 13A, C**), as indicated by immunostaining for ADA (**Figure 13A, B**) and by Nissl stain (**Figure 13C, D**). By contrast, only 1 of 4 rats injected at -4.2mm had a lesion. This posterior adjustment of injection coordinates is thus likely to substantially improve injection accuracy.

Task 4. *Test the hypothesis that ALM, but not ZOL, induces sleep by facilitating the mechanisms that underlie the transition to normal sleep.*

4a. Effects of ALM and ZOL on sleep-active brain areas.
Status: Data collection and analysis COMPLETED; manuscript in press.

4b. BF adenosine (ADO) release in response to oral ALM and ZOL.
Status: Data collection and analysis COMPLETED; manuscript in review.

4c.2 Effects of BF microinjections of ALM and ZOL on sleep/wake and neurotransmitter release in the cerebral cortex.
Status: See report below.

Progress - Task 4a: This study was described in detail in last year's Progress Report. We can now report the manuscript entitled "Homeostatic Sleep Pressure is the Primary Factor for Activation of Cortical nNOS/NK1 Neurons" was accepted for publication in the journal *Neuropsychopharmacology* on 17 July 2014.

Progress - Task 4b: Data collection and analyses for Tasks 4b and 4b.2 were completed by end of September, 2013. To form a complete story, we additionally added a subset of basal forebrain behavioral and pharmacological data from Task 3 for our manuscript. The manuscript entitled, "Hypocretin/orexin Antagonism Enhances Sleep-related Adenosine and GABA Neurotransmission in Rat Basal Forebrain" is currently in revision for publication in *Brain Structure and Function* where it has received a very favorable review. We anticipate submission of the revised version of this manuscript by 31 July 2014.

Progress - Task 4c: For Task 4c, we proposed to evaluate the effects of ALM and ZOL microinjections into the basal forebrain (BF) on sleep and wakefulness and on neurotransmitter release in the cerebral cortex (Cx). To date, no studies have reported the effect of central microinjections of hypnotics into the BF and their effects on sleep-wake behavior or neurotransmitters in brain. We proposed to use in vivo microdialysis and HPLC analyses to examine cortical adenosine (ADO), GABA, and glutamate (GLU) levels following BF microinjections of ALM, ZOL, or vehicle (VEH) combined with behavioral analyses. Task 4c was initiated mid-year in 2013 and, based on a power analysis, would require 70 rats to complete all of the n's per concentration (N=10 per group) to reach statistical significance ($p<0.05$) for this subtask. To date, a total of 23 rats contributed to the analyses for Task 4c. Several animals (N=6) had to be euthanized prior to entry into the protocol due surgical complications and thus did not contribute any data to the study. Each rat randomly received one of seven drug treatments (minimum 1 week apart with no more than two different drugs or dialysis attempts per animal) with parallel dialysis sampling in the cortex. Drug conditions (in μM) and experimental groups with the number of animals used in this study were as follows: VEH (N=8), ALM (1; N=7), ALM (0.3; N=7), ALM (0.1; N=3), ZOL (1; N=7), ZOL (0.3; N=5), and ZOL (0.1; N=4). One of seven drugs was microinjected into the animal 6 h into the dark period (ZT18), and 30 min dialysis samples (1 μL/min; CMA 12 probes) were collected to assess the effects of the drug on sleep-wake behavior and neurotransmitter levels in Cx.

Baseline EEG and EMG recordings were collected for 48 h via implanted telemetry devices concurrent with video recordings and microdialysis sampling. For all experiments, a microdialysis probe (2 mm length, 0.5 mm diameter, 20 kDa cutoff; CMA 12, CMA Microdialysis) was inserted into the cannula ~18 h prior to sample collection to allow for

neurotransmitter stabilization and perfused with aCSF at a rate of 1 µl/min. At the start of the experiment (3.5 h into the dark period; ZT 15.5), five 30 min baseline samples (1 µL/min flow rate; 30 µL total) were collected from freely-moving animals to assess basal levels of ADO, GABA, and GLU in conjunction with baseline EEG and EMG data. Following baseline collection, one of seven drug conditions was microinjected into the BF 6 h into the dark period (ZT 18) and then 12 additional 30 min samples were collected to assess the effects of the drug on sleep-wake and Cx neurotransmitter release (**Figure 14**). All samples in were collected in refrigerated fraction collectors at 4ºC and stored at -80ºC at the end of the experiment until analysis by HPLC.

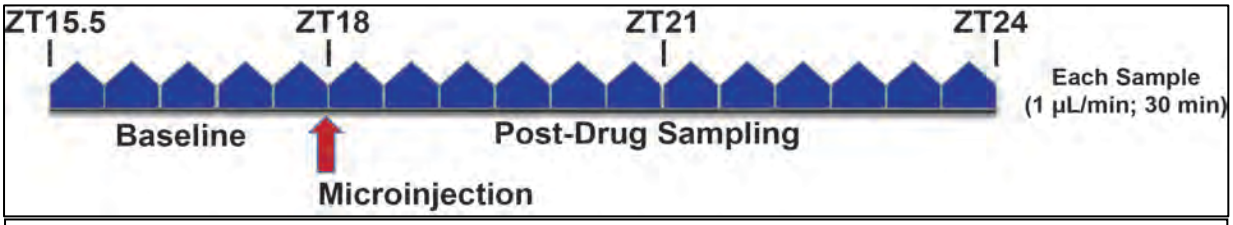

Figure 14. Schematic of the experimental design for Task 4c. Each blue pentagram box represents the collection of an individual microdialysis sample. Red arrow indicates time of microinjection at ZT18.

For HPLC quantification, the microdialysis samples were split into two vials for ADO (10 µL), GLU and GABA (20 µL) analysis. Quantification of ADO was measured by HPLC with UV detection. Samples (10 µL total volume) containing ADO were separated using mobile phase (10 mM monosodium phosphate, 7% acetonitrile, pH 4.50) pressurized through a U3000 isocratic pump with a flow of 0.8 mL/min. The dialysates flowed through a reversed-phase C18 column (150 mm ID x 4.6 mm, 2.6 µm, Phenomenex) and ADO was detected by UV at 254 nm. GLU and GABA dialysis content (20 µL total volume) were separated by HPLC with electrochemical detection (EC) using mobile phase (100 mM Na_2HPO_4, 22% MeOH, and 3.5% acetonitrile, pH 6.75) at a flow rate of 0.7 mL/min on a U3000 biocompatible isocratic pump. GLU and GABA were detected by precolumn derivatization using 2.2 mM O-phthalaldehyde and 0.8 mM 2-mercaptoethanol (β-ME) mixed by automation with the sample at 10°C for 2 min prior to injection into the HPLC. Separation was achieved through a reversed-phase C18 column (3.0 mm ID x 75 mm, 3 µm, Shiseido Capcell Pak) and electrically detected on a CouloChem III at the following potentials; E1; +250 mV, E2; +550 mV, Guard +650 mV at 45°C. Calibration curves for ADO and GLU/GABA were constructed using Chromeleon 6.8.0 software (Dionex, Corp., Sunnyvale, CA).

EEG and EMG were recorded via telemetry on a PC running Dataquest ART 3.1 (Data Sciences). All recordings were first screened for artifact and then manually scored offline in 10 s epochs as Wake, NREM, or REM sleep using NeuroScore 2.1 (DataSci™, St. Paul, MN). Any epochs that contained recording artifacts were tagged and excluded from subsequent analyses. Individual state data were quantified as time spent in each state per 30 min, 1 h, or 6 h. Latency to NR and REM onset for each animal was calculated from the time of drug injection. Bouts were defined as a minimum of 3 consecutive epochs of wake or NREM, and 2 consecutive epochs of REM sleep. NREM delta power was normalized to the average total spectral power for the 24 h baseline. Two way ANOVA and the Fisher's LSD multiple comparison test were used to determine any significant effects of drugs on neurotransmitter levels and sleep-wake behavior.

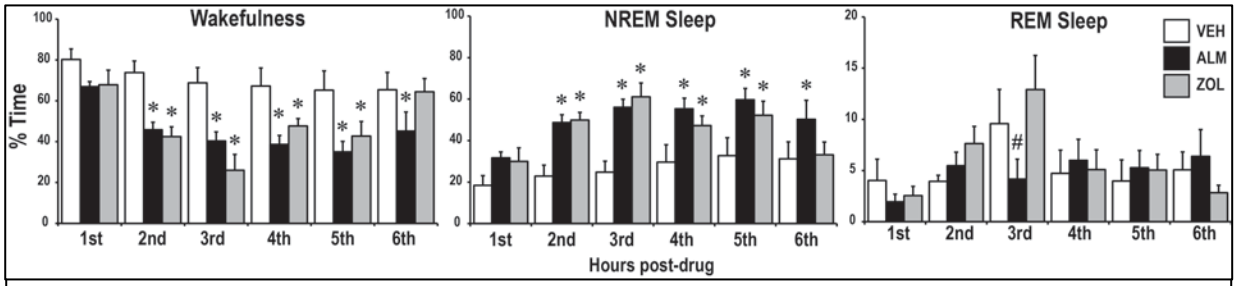

Figure 15. Effects of BF drug microinjections on percent time spent in behavioral state. ALM and ZOL caused a significant decrease ($p<0.05$) in % time spent in wake compared to VEH that persisted 6 h post-drug administration for ALM. An increase in NREM sleep with ALM and ZOL ($p<0.05$) was observed compared to VEH. * denotes significant difference from VEH. # denotes significant difference from ZOL.

Behavioral State Results: The results presented below are based on the data collected to date. There is wide variability within certain drug concentrations due to the low N for the ALM and ZOL groups, where VEH (N=8), ALM (1; N=7), ALM (0.3; N=7), ALM (0.1; N=3), ZOL (1; N=7), ZOL (0.3; N=5), and ZOL (0.1; N=4). As a result, there were insufficient N per group to perform the appropriate statistical comparisons both within and across the three drug conditions (VEH, ALM, and ZOL).

The data presented below show 3 conditions (VEH, ALM, and ZOL) for the highest dose of each drug concentration (ALM (10 ng/200 nl), ZOL (60 ng/200 nl)) and the effects on sleep-wakefulness and neurotransmitter release. BF microinjections of ALM and ZOL (**Figure 15**) significantly decreased the amount of time the animals spent in wakefulness compared to VEH controls (*$p<0.05$). This effect lasted for the 6 h post-drug administration.

Similarly, a significant increase in NREM sleep was observed in both ALM and ZOL conditions

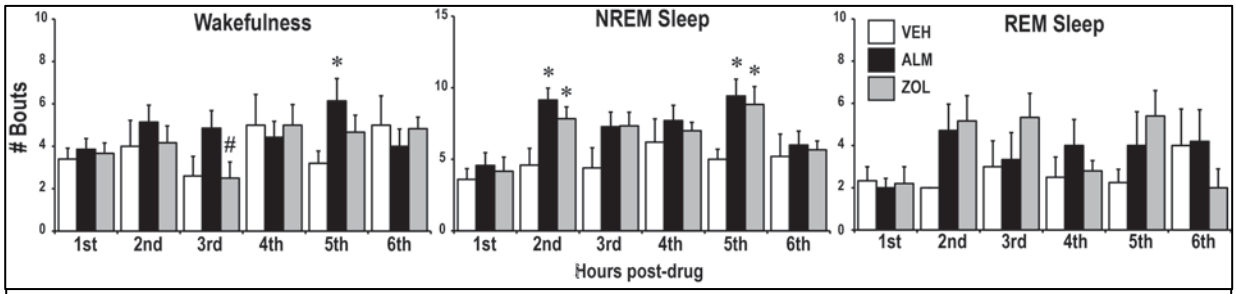

Figure 16. Effects of BF drug microinjections on bout number by behavioral state. # of Wake bouts was affected by ALM compared to VEH 5 h post drug-administration (*$p<0.05$). #$p<0.05$ denotes significant difference from ALM. # of NREM bouts also increased following ALM or ZOL at 2 and 5 h post-drug delivery (*$p<0.05$).

relative to VEH.

Wake bout frequency (**Figure 16**) was affected by ALM compared to VEH 5 h post drug-administration (*$p<0.05$). #$p<0.05$ denotes significant difference from ALM. An increase in NREM bout number was also observed with ALM and ZOL compared to VEH at 2 and 5 hrs post-drug administration (*$p<0.05$).

Although the mean duration of Wakefulness and REM sleep (**Figure 17)** was affected by ALM and ZOL compared to VEH during the 2nd, the 3rd and the 5th hours post drug (*$p<0.05$),

NREM sleep was unaffected. There was no effect on the latencies to either NREM or REM sleep.

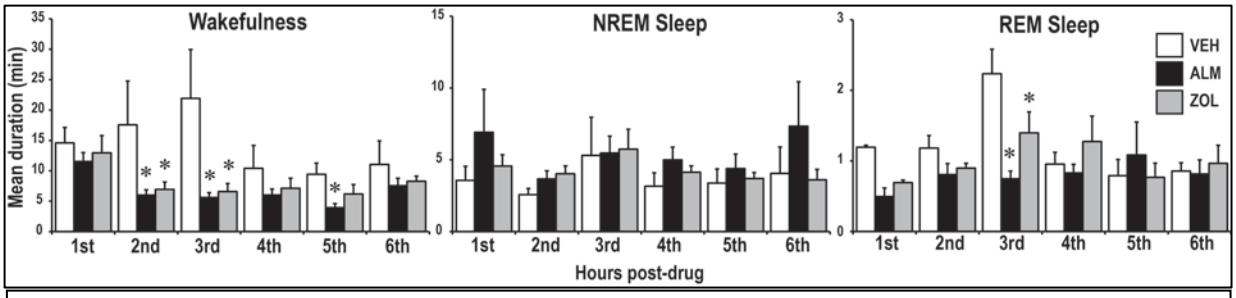

Figure 17. Effects of BF drug microinjections on mean duration for each behavioral state. Duration of Wake bouts were affected by ALM and ZOL at 2, 3 h and 5 h compared to VEH post drug-administration (*p<0.05).

Neurotransmitter Analyses and Results: Microdialysis samples were split into 2 vials and processed for both ADO, and GLU/GABA content. Two-way ANOVA revealed a significant drug x time interaction on cortical ADO release. *Post hoc* comparisons showed that ALM caused a significant increase ($p<0.05$) in cortical ADO (**Figure 18**) that persisted for hours throughout the experimental session. Microdialysis collection ended 6 h post-drug administration, at which time ADO appeared to be returning to baseline levels. On the other hand, cortical GABA levels were not significantly altered by BF microinjections of either ALM or ZOL compared to VEH. GABA levels remained stable throughout the 6 h experimental session post drug-administration. Interestingly, there appeared to be a general suppression of GLU release for several hours that began to return to basal levels by the 6th hour. *Post hoc* tests showed that ZOL caused a significant increase (#$p<0.05$) in cortical GLU relative to ALM at the end of the 6th hour post-drug. ADO is known to be inhibitory on excitatory glutamatergic neurons in vitro. These data suggest that cortical ADO may have a similar effect in vivo as our data show that ADO is significantly elevated for several hours following BF microinjections of ALM and the resulting

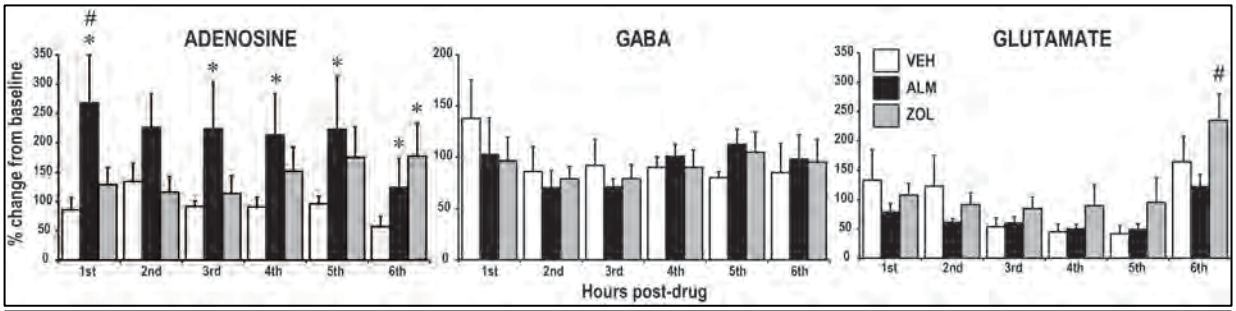

Figure 18. Effects of BF drug microinjections on neurotransmitter release in the cortex. Microinjections of ALM in the BF affected ADO release in the cortex (*p<0.05). ALM or ZOL had no effect on cortical GABA, and GLU appeared to be suppressed for several hours followed by a return to basal levels by the end of the hour 6 (#p<0.05 relative to ALM).

elevation of ADO in cortex may be providing that inhibitory influence on the surrounding glutamatergic neurons.

This study demonstrates that microinjection of ALM into the BF significantly increases the amount of time spent in NREM sleep at the expense of wakefulness (as seen by its effects on wake duration) similar to oral administration, as previously described (Dugovic et al., 2009;

Morairty et al., 2012). Microinjection of ZOL at the same dose also increased the percent of time spent in NREM sleep comparable to ALM. Microinjection of ALM increased the frequency of NREM bouts compared to VEH. Microinjection of ALM and ZOL decreased Wake bout duration compared to VEH. Microinjection of ALM promoted a sustained increase in ADO in the cerebral cortex over the 6 h recording during the dark period, whereas ZOL had no effect on ADO levels. Other cortical neurotransmitters such as GABA and GLU remained unaffected at the drug doses delivered to the BF. Consistent with these observations, the hypocretins (orexins) are known to modulate diverse physiological processes such as cognitive function and alertness.

The results of this study indicate that microinjection of ALM can induce sleep similar to oral delivery and facilitates the mechanisms that underlie the transition to normal sleep. ALM acts through blockade of post-synaptic Hcrt receptors, thereby disfacilitating excitation in the BF whereas ZOL, a benzodiazepine receptor agonist, affects a Cl^- channel on the $GABA_A$ receptor, resulting in hyperpolarization and general somnolence. In addition, systemic and local delivery of ALM (unlike ZOL) enhances ADO in both Cx and BF (manuscript under review) suggesting that the sleep-promoting effect of ADO may be via inhibition of Hcrt regulation of sleep-wakefulness.

Task 6: *Utilize optogenetics and in vivo physiology to compare the neural circuitry underlying ALM-induced vs. ZOL-induced sleep.*

6a. Determine whether activation of the Hcrt system is sufficient to induce arousal in the presence of ALM vs. ZOL.

6b. Determine whether ALM affects the activity of subcortical sites downstream from the Hcrt neurons.

6c. Determine how ALM and ZOL affect the activity of cortical neurons.

Technology Development: As described in last year's Progress Report, it was necessary to update the *In Vivo* Cellular Neurophysiology Laboratory before undertaking the experiments for Task 6 so that we could reliably perform optogenetic stimulation in freely-behaving mice across different recording sessions. This year, we made further technical refinements to our setup by utilizing commercially-produced bilateral fiber optic implants and a rotary joint that enabled light to be delivered to a mouse without constraining its movements (**Figure 19**). The bilateral implants depicted in Figure 19B are stereotactically implanted 1 mm above the hypocretin (Hcrt) field at 1mm lateral, 1.5 mm A-P and 3.5 mm D-V. To facilitate Aim 6b, we also acquired a microdrive that will allow us to position a Neuronexus array of 4 tetrodes that connects to the head stage of a Tucker Davis amplifier, thereby enabling us to record single neurons in deep brain areas while animals are freely moving (**Figure 20A**). To complement the electrophysiological measurements to be conducted in Aims 6b and 6c, we are also developing the capability to conduct calcium imaging in deep brain areas using the technology created by Inscopix, Inc. This technology consists of a miniature (~2 g) fluorescence microscope that can be mounted on the skull of a mouse and a microendoscope lens that can be implanted in the brain parenchyma. When coupled with genetically-encoded calcium indicators such as GCaMP6 expressed in local brain areas using a viral vector, the activity of hundreds of neurons can be visualized simultaneously (**Figure 20B and C**).

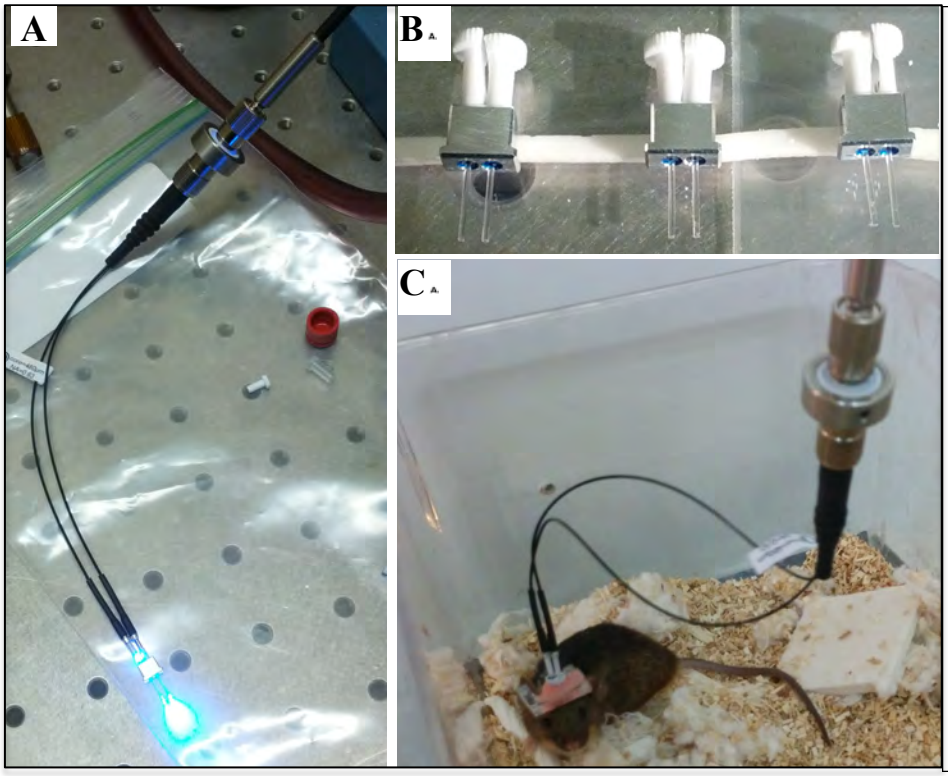

Figure 19. **A**) A LED based light engine (Lumencor) connects to a rotary joint which in turn connects to a patch cord that divides the light into two 0.5 mm fibers that can be attached to a bilateral fiber optic implant delivering 10 mW of light intensity at the output of each implanted fiber. **B**) Bilateral optical fiber implants (0.5 mm diameter). **C**) Implanted mouse in its home cage connected to the fiber optics for *in vivo* stimulation. The cage is placed over a DSI receiver that register EEG & EMG.

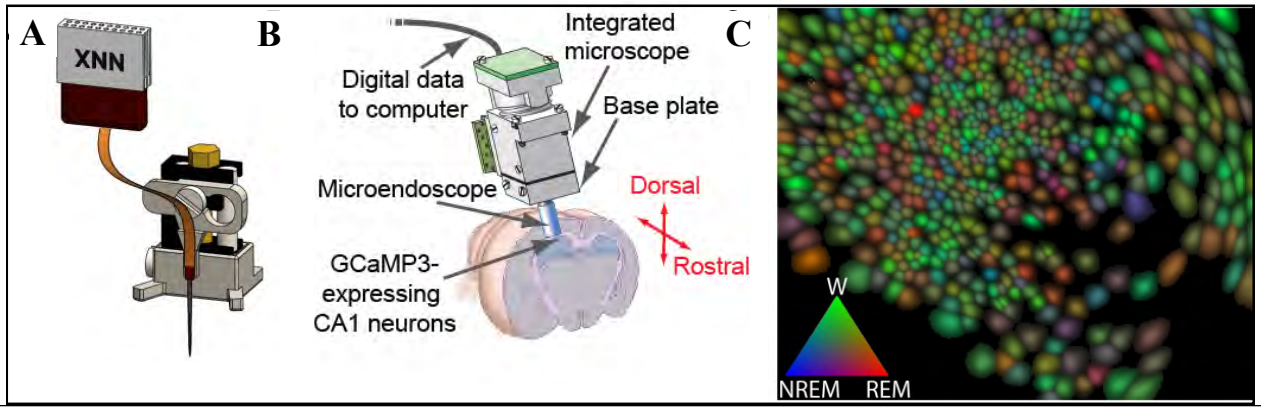

Figure 20. **A**) Schematic of the Neuronexus microdrive. **B**) Schematic of the Inscopix miniature fluorescence microscope. **C**) Example of hippocampal neurons identified with the Inscopix microscope and color-coded according to their activity rate during NREM sleep, REM sleep and Wake.

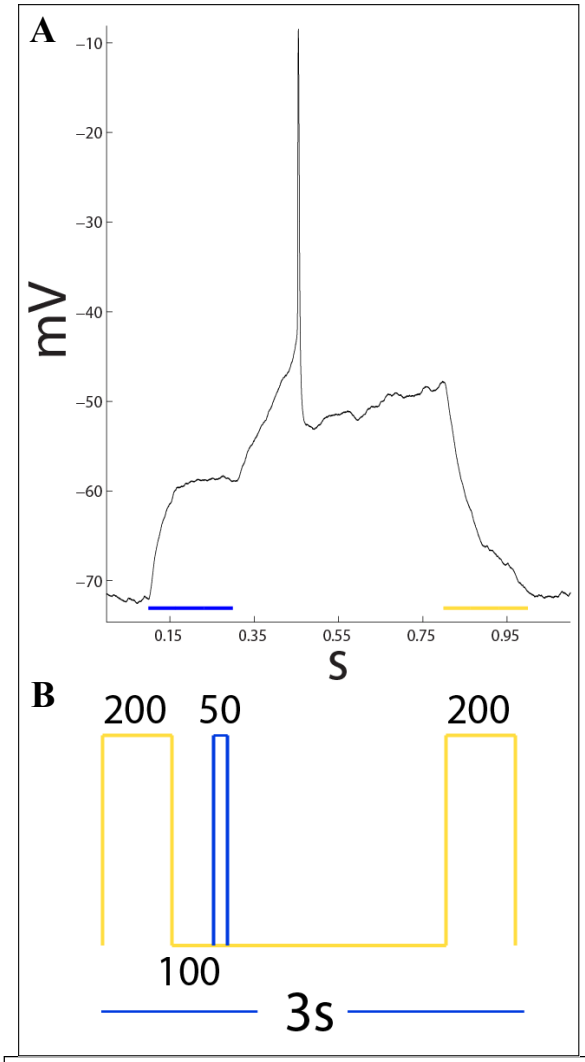

Figure 21. **A**. Depolarization of an Hcrt neuron from an *orexin-tTA; Tet-O ChR2(C128S)* mouse when illuminated by 100 ms pulse of blue and yellow light. **B**. For *in vivo* experiments, a 200 ms yellow pulse was followed by a 50 ms blue pulse and, 2650 ms later, by another 200 ms yellow pulse.

Progress - Task 6a: *In vitro* recordings of Hcrt neurons of *orexin-tTA; Tet-O ChR2(C128S)* mice show that a pulse of blue light depolarizes these neurons (**Figure 21**). *ChR2(C128S)* encodes a "step function opsin" (SFO) in which sodium channels remain open after blue light stimulation until closed by yellow light stimulation. Thus, in **Figure 21A**, V_m remains depolarized after the blue pulse until a yellow pulse closes the channels. To prevent desensitization of the SFO and to ensure that all channels were closed before delivering the pulse of blue light, we stimulated mice with a 50 ms pulse of blue light flanked by 200 ms yellow pulses (see **Figure 21B**) once every 4 min. To control for a possible effect of light illumination, we interleaved this stimulus pattern with pure yellow stimulation with the same pattern, i.e., the yellow 50 ms pulse was repeated once every 4 min. Thus, mice received either blue light flanked by yellow stimulation or a pure yellow stimulus every 2 min.

Mice (N=4) were implanted with the fiber optic implants described above and with a DSI telemetry transmitter to enable EEG and EMG recording. Recordings started at ZT4 and animals were dosed with either vehicle (Veh) or 300 mg/Kg almorexant (ALM) at ZT5. The volume injected was 0.15 ml IP. One hour later, optogenetic stimulation commenced at ZT6 for 1 h and an additional hour of EEG/EMG was recorded.

Figure 22 shows the raw EEG, EMG and light stimulus signal when mice were dosed either

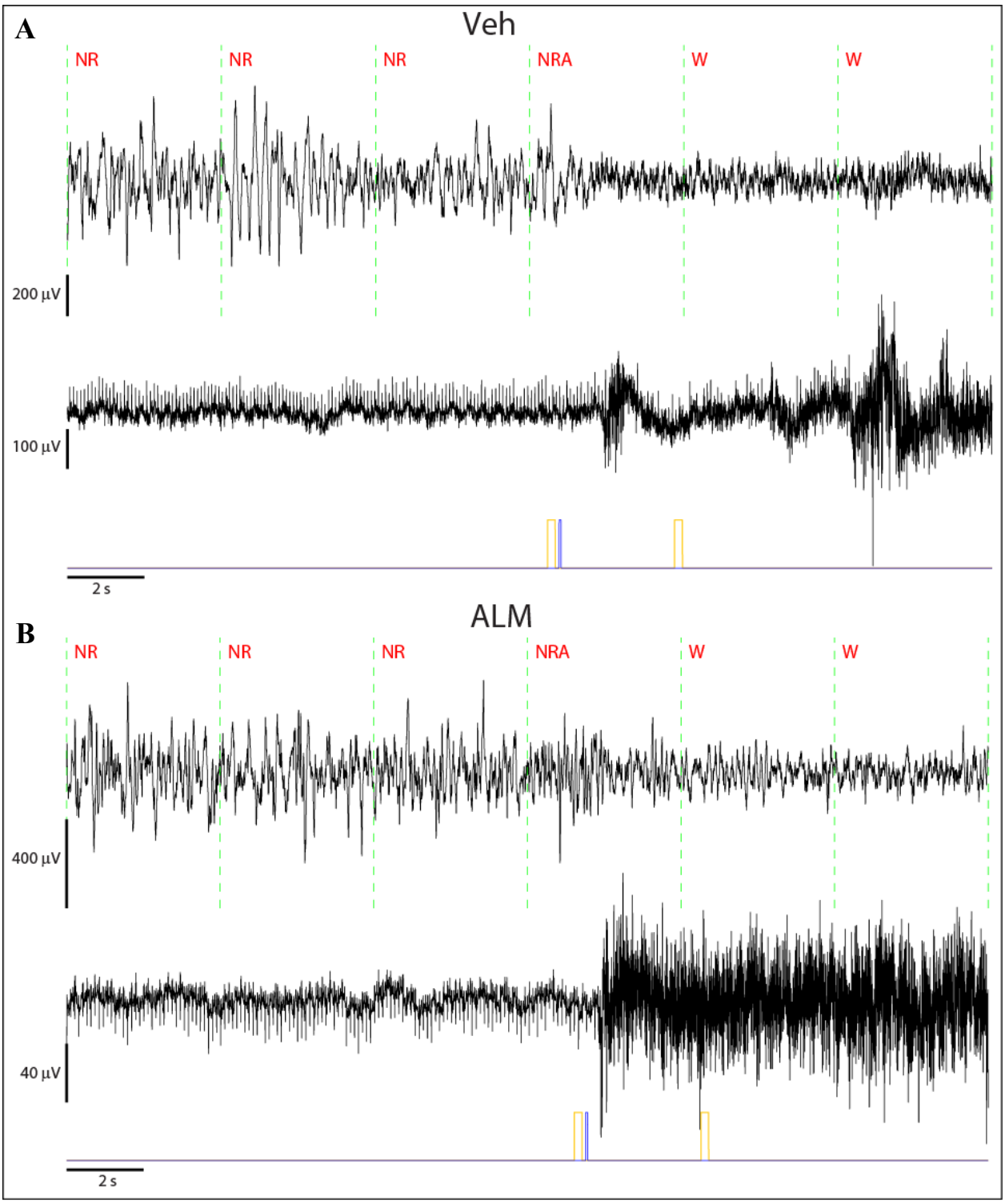

Figure 22. EEG/EMG recording of a mouse dosed with either Veh (**A**) or ALM (**B**) during optogenetic stimulation of Hcrt neurons. In each panel, the upper trace shows the EEG, middle trace is the EMG, and lower trace shows the light stimulus. 4 sec epochs were scored as either Wake (W), NREM sleep (NR) or NREM with artifact (NRA).

with Veh or ALM. In both treatment conditions, mice tended to awaken after the blue pulse

either briefly or for prolonged periods, as depicted in **Figure 22**. Note that, in both cases, the stimulation and awakening occurred within the same 4s epoch. When the stimulus was delivered during NREM sleep, animals woke up within 4 s after the blue pulse in 79% and 76% of the cases for Veh and ALM, respectively.

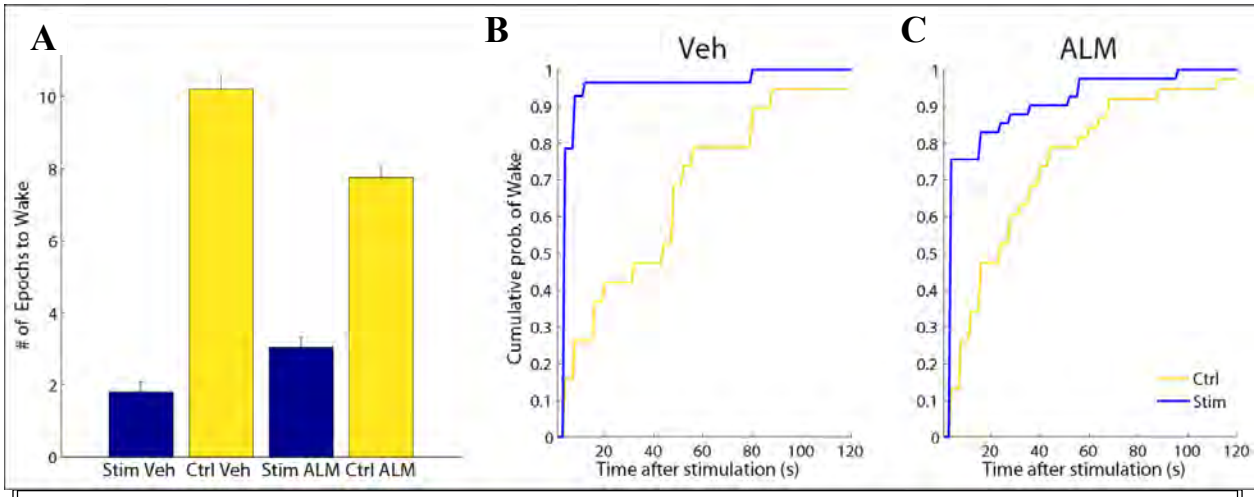

Figure 23. **A.** Number of 4s epochs elapsed after the light pulse until awakening occurred. Pulses were delivered during NREM sleep. **B, C.** Cumulative probability of wake after illumination when stimulated with blue light (stim) or with control (Ctrl) yellow light after Veh (**B**) or ALM (**C**) treatment.

Figure 23A shows the average number of epochs that elapsed from stimulation during NREM sleep until mice (N=4) awoke. In Veh-injected mice, the latencies to awakening were 1.8±0.3 and 10.2±0.4 epochs for 50 ms blue and yellow light pulses, respectively. For ALM-treated mice, the latencies to awakening were 3±0.3 and 7.8±0.4 epochs for 50 ms blue and yellow light pulses, respectively. Blue and yellow stimulation caused a significant difference (U test, $P<0.01$) but, within the same stimulus type, there was no difference between treatments in the 4 animals tested to date. **Figure 23B and C** show the cumulative probability of wake after stimulation with either blue (Stim) or yellow (Ctrl) light after Veh or ALM treatment. The fast rise of the Stim curve indicates that, in the large majority of cases, wake occurred in the next epoch after blue stimulation irrespective whether the mice had been treated with either Veh or ALM. After the Ctrl stimulus, the latencies to awakening are much longer, suggesting that illumination *per se* does not induce awakening. The fast time course of the awakening and the absence of a clear difference in the presence of the Hcrt antagonist ALM suggests that the observed arousals are mediated by glutamate release from Hcrt neurons projecting to wake-promoting areas such as the locus coeruleus (LC).To further characterize the changes in EEG upon Hcrt activation, we performed time-frequency analysis using 2 s windows. To calculate the average power in the standard EEG frequency bands, the FFT was shifted in 100 ms steps around the time of stimulation during NREM sleep. **Figure 24** shows that, for all bands below 60 Hz, optogenetic stimulation produced the decrease in spectral power amplitude that is expected during a transition from sleep to wake. This type of plot allows us to visualize the effect of Hcrt activation with greater time resolution in contrast to the coarse-grained analysis afforded by the 4s epoch classification shown in **Figure 22**. For both ALM and Veh, the change in spectral power is almost instantaneous at the time of stimulation, as reported when LC neurons were activated by direct optogenetic excitation (Carter et al 2010). This result supports the hypothesis that Hcrt neuron-mediated glutamate release evoked firing of LC neurons that, in turn, evoked a

general arousal.

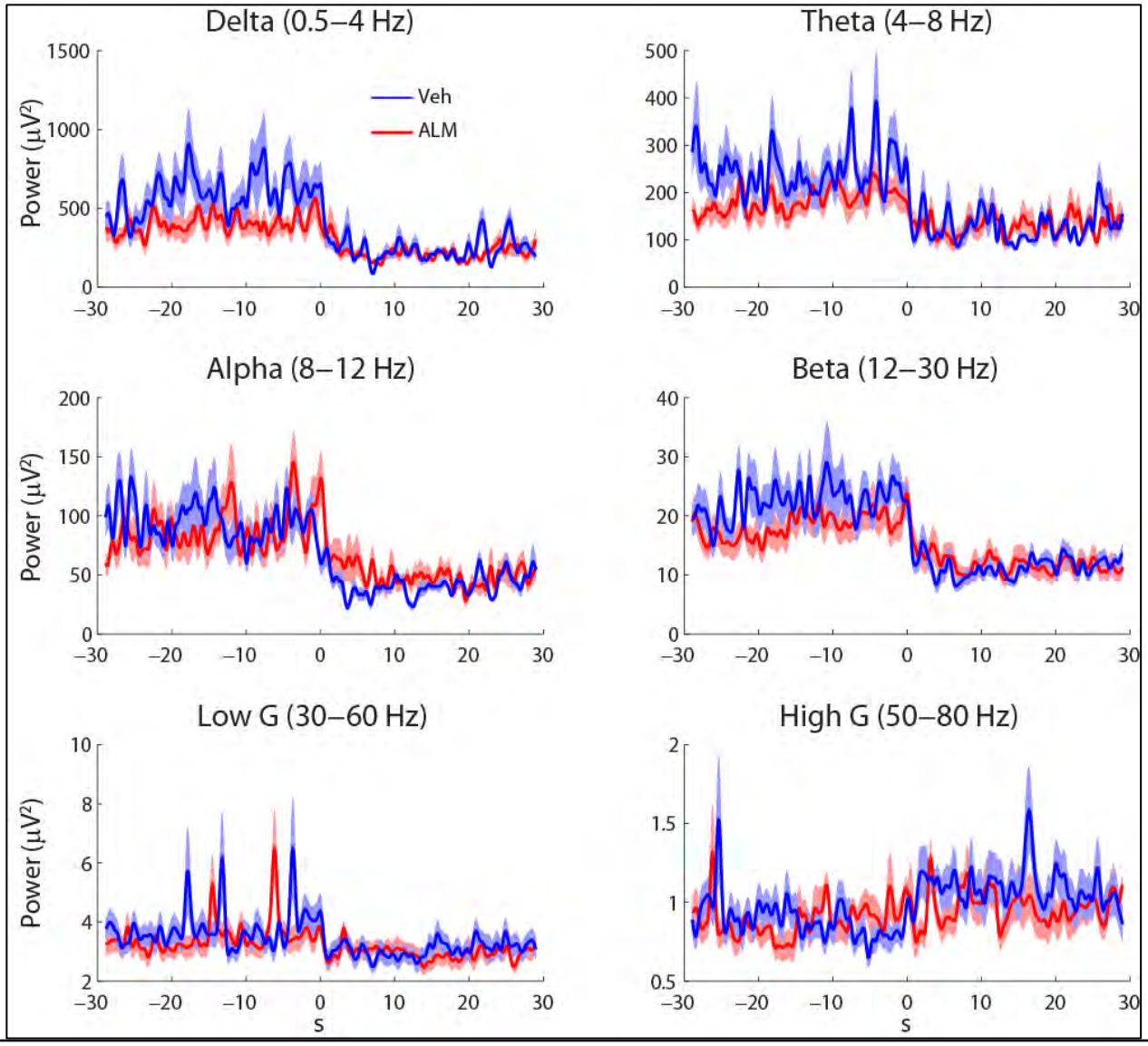

Figure 24. Time-frequency analysis for the average power in the standard EEG spectral bands. No significant difference was observed between ALM and Veh. Traces are centered on the time of blue light stimulation and the average power and SEM is depicted.

Plans for Year 6:

Task 2c: Data analysis for the rPVT study to be completed and manuscript to be submitted for publication.

Task 3a: The last few Fos double labeling experiments will be completed. Primary histology experiments have been completed for all markers, but staining needs to be repeated for a few animals for the ADA and Hcrt markers to ensure that all subregions have a sufficient number of cells scored to ensure an accurate analysis. Additionally, EEG/EMG analysis will be completed and the results correlated with Fos expression patterns. Once all experiments have been completed and analyzed, the results of this study will be prepared for publication.

Task 3b: Analysis for the second (locus coeruleus) lesion studies completed; a manuscript will be submitted for publication by 12/31/14. Data collection and analysis for the third (tuberomammillary nucleus) lesion study, which will require an additional 8 rats, will be completed.

Task 6a: We will complete Task 6a, optogenetic activation of Hcrt neurons in *orexin-tTA; Tet-O ChR2(C128S)* mice, and study the effects on sleep architecture at ZT 6 and 18 and after treatment with either Veh, ALM or Zol.

Tasks 6b and 6c: We will continue efforts to complete these tasks.

KEY RESEARCH ACCOMPLISHMENTS

- Aims 2a, 2b, and 4a completed and published.
- Manuscript describing results of Aims 3b.1 and 4b submitted and in revision.
- Data collection for Aims 2c and 3b.2 completed and data analysis ongoing.
- Data collection and analysis of Aim 3a continued.
- Data collection for Aims 3b.3, 4c and 6a initiated. In Task 6a, performed optogenetic excitation in freely behaving *orexin-tTA; Tet-O ChR2(C128S)* mice, which seem to tolerate the implant and the optogenetic stimulation without noticeable side effects.
- Initial results from Task 6a indicate that Hcrt neuron activation can cause a fast arousal that does not seem to be mediated by release of the Hcrt peptides as is not blocked in the presence of the Hcrt antagonist ALM.
- Implementation of a microdrive and a Neuronexus probe for recording multiunit activity in deep brain areas in Task 6b.
- Incorporated use of the Inscopix technology to record the activity of populations of neurons in specific brain areas.

REPORTABLE OUTCOMES

J. Vazquez-DeRose, A. Nguyen, S. Gulati, T. Mathew, and T. S. Kilduff (2013). Microinjections of the hypocretin antagonist almorexant vs. GABAergic agonist zolpidem in basal forebrain show differential effects on cortical adenosine levels in freely-moving rats. Program No. 478.11. *2013 Neuroscience Meeting Planner*. San Diego, CA: Society for Neuroscience, 2013. Online.

W. Lincoln, J Palmertson, T.C. Neylan, T.S. Kilduff, S.R. Morairty (2013). Zolpidem impairs attention/motivation in the rodent Psychomotor Vigilance Task more than almorexant. Program No. 658.24. *2013 Neuroscience Meeting Planner*. San Diego, CA: Society for Neuroscience, 2013. Online.

Morairty SR, Wilk A. Lincoln W, Neylan TC, and Kilduff TS. (2014). The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats. *Front. Neurosci*. doi: 10.3389/fnins.2014.00003

Dittrich L, Morairty SR, Warrier D and Kilduff TS. Homeostatic sleep pressure is the primary factor for activation of cortical nNOS/NK1 neurons. *Neuropsychopharmalogy*, in press.

Vazquez-DeRose J, Schwartz MD, Nguyen AT, Warrier DR, Gulati S, Mathew TK, Neylan TC, and Kilduff TS. Hypocretin/orexin antagonism enhances sleep-related adenosine and GABA neurotransmission in rat basal forebrain. *Brain Structure and Function*, provisionally accepted.

CONCLUSION

During Year 5, one paper was published (Morairty et al. 2014), another has been accepted for publication (Dittrich et al., in press) and a third is in resubmission (Vazquez-DeRose et al., submitted). Data collection for Aims 2c and 3b.2 have been completed and data analysis ongoing; manuscripts will be written and submitted during Year 6. Data collection and analysis of Aim 3a in nearing completion; an abstract summarizing this work has been submitted for presentation at the 2014 Society for Neuroscience meeting. Data collection for Aims 3b.3, 4c and 6a have been initiated. The overall results obtained to date are consistent with the hypothesis that the hypocretin/orexin antagonist ALM produces less functional impairment than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems.

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Morairty SR, Revel FG, Malherbe P, Moreau JL, Valladao D, Wettstein JG, Kilduff TS, Borroni E (2012). Dual hypocretin receptor antagonism is more effective for sleep promotion than antagonism of either receptor alone. *PLoS One* 7(7):e39131. doi: 10.1371/journal.pone.0039131.

APPENDICES

J. Vazquez-DeRose, A. Nguyen, S. Gulati, T. Mathew, and T. S. Kilduff (2013). Microinjections of the hypocretin antagonist almorexant vs. GABAergic agonist zolpidem in basal forebrain show differential effects on cortical adenosine levels in freely-moving rats. Program No. 478.11. *2013 Neuroscience Meeting Planner.* San Diego, CA: Society for Neuroscience, 2013. Online.

W. Lincoln, J Palmertson, T.C. Neylan, T.S. Kilduff, S.R. Morairty (2013). Zolpidem impairs attention/motivation in the rodent Psychomotor Vigilance Task more than almorexant. Program No. 658.24. *2013 Neuroscience Meeting Planner.* San Diego, CA: Society for Neuroscience, 2013. Online.

Morairty SR, Wilk A. Lincoln W, Neylan TC, and Kilduff TS. (2014). The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats. *Front. Neurosci.* doi: 10.3389/fnins.2014.00003

Dittrich L, Morairty SR, Warrier D and Kilduff TS. Homeostatic sleep pressure is the primary factor for activation of cortical nNOS/NK1 neurons. *Neuropsychopharmacology*, in press.

Presentation Abstract

Program#/Poster#:	658.24/BBB16
Presentation Title:	Zolpidem impairs attention/motivation in the rodent psychomotor vigilance task more than almorexant
Location:	Halls B-H
Presentation time:	Tuesday, Nov 12, 2013, 4:00 PM - 5:00 PM
Topic:	++E.08.e Sleep: Systems and behavior
Authors:	**W. LINCOLN**[1], J. PALMERSTON[1], T. NEYLAN[2], T. KILDUFF[1], *S. R. MORAIRTY[1]; [1]Ctr. for Neurosci., SRI Int'l, MENLO PARK, CA; [2]UCSF/SFVAMC, San Francisco, CA
Abstract:	The dual hypocretin receptor (HcrtR) antagonist almorexant (ALM) has potent hypnotic actions but less is known about its effects on performance. Since Hcrt antagonists produce sleep by disfacilitation of wake-promoting systems whereas benzodiazepine receptor agonists (BzRAs) such as zolpidem (ZOL) induce sleep through a generalized inhibition of neural activity, we hypothesized that ALM would produce less functional impairment than ZOL. We have previously shown that rats tested in spatial reference memory or spatial working memory tasks in a water maze show no impairment following ALM whereas significant impairment was evident following ZOL. Here, we tested the effects of ALM and ZOL on the Rodent Psychomotor Vigilance Task (rPVT), a sensitive test of attention and motivation. 10 rats were implanted with telemetry devices for recording EEG and EMG. The effects of ALM and ZOL on attention/motivation administered in the middle of the active period were assessed at 2 sleep-promoting concentrations (30 & 100 mpk, po) following undisturbed and sleep deprived (SD, 6 h prior to dosing) conditions. 90 min following dosing, trained, water-restricted rats were placed in operant chambers with infrared detection beams in front of the water dispenser. rPVT testing consisted of a stimulus light (duration of 0.5 s) followed by a 3 s response period. The inter-trial interval varied between 3-7 s. Errors resulted in a cued 10 s “time out” period. Performance measures were 1) response latencies (RL), 2) correct responses (CR), 3) omissions (OM), and 4) premature errors (PE).

Impaired performance is indicated by increases in RL, OM and PE and decreases in CR.
SD had a relatively small but significant effect on performance following VEH: RL decreased (96.2%), CR decreased (95.5%) and OM increased (146.1%) while PE decreased (95.1%) following SD+90 min recovery compared to baseline. Following ALM at 30 mpk compared to VEH, OM and RL decreased (51.7 & 96.2%; indicative of increased performance) while CR decreased and PE increased (indicative of impaired performance). ZOL at 30 mpk decreased performance markedly: RL increased (131.3%), CR decreased (30.4%) and OM increased (724.1%) while PE decreased (36.9%) compared to VEH. However, performance decreased significantly following both drugs at 100 mpk, particularly with ZOL. Following ALM at 100 mpk, RL and OM increased (150.6 & 556.3%) and CR and PE decreased (42.8 & 58.0%). Following ZOL at 100 mpk, RL and OM increased (126.6 & 855.6%) and CR and PE decreased (9.2 & 26.0%).
These results are consistent with the hypothesis that less functional impairment results from HcrtR antagonism than from BzRA-induced inhibition.

Disclosures: **W. Lincoln:** None. **S.R. Morairty:** None. **J. Palmerston:** None. **T. Kilduff:** None. **T. Neylan:** None.

Keyword(s): ATTENTION

SLEEP DEPRIVATION

MOTIVATION

Support: USAMRMC grant W81XWH-09-2-0081

Internal SRI funds

Presentation Abstract

Program#/Poster#:	478.11/JJJ25
Presentation Title:	Microinjections of the hypocretin antagonist almorexant vs. GABAergic agonist zolpidem in basal forebrain show differential effects on cortical adenosine levels in freely-moving rats
Location:	Halls B-H
Presentation time:	Monday, Nov 11, 2013, 3:00 PM - 4:00 PM
Topic:	++E.08.c. Sleep: Molecular, cellular, and pharmacology
Authors:	***J. VAZQUEZ-DEROSE**[1], A. NGUYEN[1], S. GULATI[1], T. MATHEW[1], T. C. NEYLAN[2], T. S. KILDUFF[1]; [1]Ctr. for Neuroscience, Biosci. Div., SRI Intl., MENLO PARK, CA; [2]VA Med. Center/NCIRE, UCSF San Francisco, San Francisco, CA
Abstract:	Hypocretin (Hcrt-1 and Hcrt-2) peptides are well-known to regulate sleep and alertness and send projections to the basal forebrain (BF), an area critical for promoting wakefulness. The BF contains a heterogeneous mix of neurons that send diverse projections important for cortical arousal. Almorexant (ALM) is a dual Hcrt receptor antagonist that reversibly blocks signaling of both Hcrt receptors, whereas Zolpidem (ZOL) is a benzodiazepine receptor agonist affecting Cl- channels. Previous studies have shown that oral delivery of ALM elicits somnolence without cataplexy and, in rat, decreases active wake and increases the time spent in non-rapid eye movement (NREM) and (REM) sleep with differential effects on various neurotransmitter systems. To date, no studies have reported the effects of central microinjections of ALM or ZOL and its effect on behavior or transmitter levels in brain. We used in vivo microdialysis and HPLC analysis to examine cortical adenosine (ADO) levels following BF microinjections of ZOL (0.6 μg/0.2 μl), ALM (1.0 μg/0.2 μl), or VEH (aCSF) combined with behavioral analyses. Preliminary analyses revealed a significant main effect of drug on ADO levels. Post-hoc comparisons showed that ALM microinjected in to the BF (n=3 rats; * $p<0.05$) caused a significant increase in cortical ADO that lasted up to 6 h post microinjection compared to VEH control (n=3). Conversely, administration of ZOL (n=3) to the BF significantly decreased cortical ADO levels (# $p<0.05$) compared to VEH and ALM. These results provide novel evidence for dynamic

neurochemical changes underlying Hcrt modulation of sleep-wakefulness.

Disclosures: **J. Vazquez-Derose:** None. **A. Nguyen:** None. **S. Gulati:** None. **T.C. Neylan:** None. **T. Mathew:** None. **T.S. Kilduff:** None.

Keyword(s): HYPOCRETIN

MICRODIALYSIS

ADENOSINE

Support: W81XWH-09-2-0081

frontiers in NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
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The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats

Stephen R. Morairty[1]*, Alan J. Wilk[1], Webster U. Lincoln[1], Thomas C. Neylan[2] and Thomas S. Kilduff[1]

[1] SRI International, Center for Neuroscience, Biosciences Division, Menlo Park, CA, USA
[2] Department of Psychiatry, SF VA Medical Center/NCIRE/University of California, San Francisco, CA, USA

Edited by:
Christopher J. Winrow, Merck, USA

Reviewed by:
Collin Park, University of South Florida, USA
Michihiro Mieda, Kanazawa University, Japan

***Correspondence:**
Stephen R. Morairty, SRI International, 333 Ravenswood Ave., Menlo Park, CA, USA
e-mail: stephen.morairty@sri.com

The hypocretin receptor (HcrtR) antagonist almorexant (ALM) has potent hypnotic actions but little is known about neurocognitive performance in the presence of ALM. HcrtR antagonists are hypothesized to induce sleep by disfacilitation of wake-promoting systems whereas $GABA_A$ receptor modulators such as zolpidem (ZOL) induce sleep through general inhibition of neural activity. To test the hypothesis that less functional impairment results from HcrtR antagonist-induced sleep, we evaluated the performance of rats in the Morris Water Maze in the presence of ALM vs. ZOL. Performance in spatial reference memory (SRM) and spatial working memory (SWM) tasks were assessed during the dark period after equipotent sleep-promoting doses (100 mg/kg, po) following undisturbed and sleep deprivation (SD) conditions. ALM-treated rats were indistinguishable from vehicle (VEH)-treated rats for all SRM performance measures (distance traveled, latency to enter, time within, and number of entries into, the target quadrant) after both the undisturbed and 6 h SD conditions. In contrast, rats administered ZOL showed impairments in all parameters measured compared to VEH or ALM in the undisturbed conditions. Following SD, ZOL-treated rats also showed impairments in all measures. ALM-treated rats were similar to VEH-treated rats for all SWM measures (velocity, time to locate the platform and success rate at finding the platform within 60 s) after both the undisturbed and SD conditions. In contrast, ZOL-treated rats showed impairments in velocity and in the time to locate the platform. Importantly, ZOL rats only completed the task 23–50% of the time while ALM and VEH rats completed the task 79–100% of the time. Thus, following equipotent sleep-promoting doses, ZOL impaired rats in both memory tasks while ALM rats performed at levels comparable to VEH rats. These results are consistent with the hypothesis that less impairment results from HcrtR antagonism than from $GABA_A$-induced inhibition.

Keywords: hypocretins/orexins, cognitive impairment, memory impairment, hypnotics, water maze, spatial reference memory, spatial working memory, EEG

INTRODUCTION

Insomnia is a highly prevalent condition affecting 10–30% of the general population; (NIH, 2005; Roth, 2007; Mai and Buysse, 2008). Sleep loss and sleep disruption can lead to a degradation of neurocognitive performance as assessed by objective and subjective measures (Wesensten et al., 1999; Belenky et al., 2003; Lamond et al., 2007). Prescription sleep medications are often used to treat insomnia and obtain desired amounts of sleep. Presently, nonbenzodiazepine, positive allosteric modulators of the $GABA_A$ receptor such as zolpidem (ZOL) are the most widely prescribed hypnotic medications. Although known to induce sleep, these compounds have been shown to significantly impair psychomotor and memory functions in rodents (Huang et al., 2010; Uslaner et al., 2013; Zanin et al., 2013), non-human primates (Makaron et al., 2013; Soto et al., 2013; Uslaner et al., 2013) and humans (Balkin et al., 1992; Wesensten et al., 1996, 2005; Mattila et al., 1998; Mintzer and Griffiths, 1999; Verster et al., 2002; Storm et al., 2007; Otmani et al., 2008; Gunja, 2013). Such impairment can be particularly troubling when there is an urgent need for highly functional performance in the presence of drug such as with first responders, military personnel, and caregivers. Further, complex behaviors during the sleep period (e.g., eating, cooking, driving, conversations, sex) have been associated with these medications (Dolder and Nelson, 2008). Therefore, more effective hypnotics are needed that facilitate sleep that is easily reversible in the event of an unexpected awakening that demands unimpaired cognitive and psychomotor performance.

Recently, antagonism of the hypocretin (Hcrt; also called orexin) receptors has been identified as a target mechanism for the next generation of sleep medications (Brisbare-Roch et al., 2007; Dugovic et al., 2009; Whitman et al., 2009; Hoever et al., 2010, 2012a,b; Coleman et al., 2012; Herring et al., 2012; Winrow et al., 2012; Betschart et al., 2013). The Hcrt system is well known to play an important role in the maintenance of wakefulness (de Lecea, 2012; Inutsuka and Yamanaka, 2013; Mieda and Sakurai, 2013; Saper, 2013). Hcrt fibers project throughout the central nervous system (CNS), with particularly dense projections and receptor expression found in arousal centers including the locus

coeruleus, the tuberomammilary nucleus, dorsal raphe nuclei, laterodorsal tegmentum, pedunculopontine tegmentum, and the basal forebrain (Peyron et al., 1998; Marcus et al., 2001). The excitatory effects of the Hcrt peptides on these arousal centers is hypothesized to stabilize and maintain wakefulness. Therefore, blockade of the Hcrt system should disfacilitate these arousal centers, creating conditions that are permissive for sleep to occur.

The current study tests the hypothesis that the dual Hcrt receptor antagonist almorexant (ALM) produces less functional impairment than ZOL. The rationale that underlies this hypothesis is that ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. We tested this hypothesis using tests of spatial reference memory (SRM) and spatial working memory (SWM) in the Morris Water Maze. Although the concentrations of ALM and ZOL administered prior to these tests were equipotent in hypnotic efficacy, the performance of rats treated with ALM were superior to that of rats treated with ZOL.

MATERIALS AND METHODS

ANIMALS

One hundred fifty three male Sprague Dawley rats (300 g at time of purchase; Charles River, Wilmington, MA) were distributed among the 12 groups as described in **Table 1**. All animals were individually housed in temperature-controlled recording chambers (22 ± 2°C, 50 ± 25% relative humidity) under a 12:12 light/dark cycle with food and water available *ad libitum*. All experimental procedures were approved by SRI International's Institutional Animal Care and Use Committee and were in accordance with National Institute of Health (NIH) guidelines.

SURGICAL PROCEDURES

Rats were instrumented with sterile telemetry transmitters (F40-EET, Data Sciences Inc., St Paul, MN) as previously described (Morairty et al., 2008, 2012; Revel et al., 2012, 2013). Briefly, under isoflurane anesthesia, transmitters were placed intraperitoneally and biopotential leads were routed subcutaneously to the head and neck. Holes were drilled into the skull at 1.5 mm anterior to bregma and 1.5 mm lateral to midline, and 6 mm posterior to bregma and 4 mm lateral to midline on the right hemisphere. Two biopotential leads used as EEG electrodes were inserted into the holes and affixed to the skull with dental acrylic. Two biopotential leads used as EMG electrodes were positioned bilaterally through the nuchal muscles.

IDENTIFICATION OF SLEEP/WAKE STATES

After at least 3 weeks post-surgical recovery, EEG, and EMG were recorded via telemetry using DQ ART 4.1 software (Data Sciences Inc., St Paul, MN). Following completion of data collection, the EEG, and EMG recordings were scored in 10 s epochs as waking (W), rapid eye movement sleep (REM), or non-rapid eye movement sleep (NREM) by expert scorers blinded to the treatments using NeuroScore software (Data Sciences Inc., St Paul, MN). Sleep latency was defined as the first 60 s of continuous sleep following drug administration. Recordings were started at Zeitgeber time (ZT) 12 (lights off) and continued until animals performed the water maze tests.

Table 1 | The number of rats tested for each of the 12 experimental groups.

Test	No SD			6 h SD		
	VEH	ALM	ZOL	VEH	ALM	ZOL
Reference memory	14	13	17	16	16	8
Working memory	11	12	12	12	11	11

SLEEP DEPRIVATION PROCEDURES

Animals were sleep deprived (SD) from ZT12-18 by progressive manual stimulation concurrent with EEG and EMG recording. The rats were continuously observed and, when they appeared to attempt to sleep, progressive interventions were employed to keep them awake: removal of cage tops, tapping on cages, placement of brushes inside the cage, or stroking of vibrissae or fur with an artist's brush.

DRUGS

Almorexant (ALM; ACT-078573), was synthesized at SRI International (Menlo Park, CA. USA) according to the patent literature. Zolpidem (ZOL) was a gift from Actelion Pharmaceuticals Ltd. For the SWM task, rats were dosed with ALM (100 mg/kg, p.o.), ZOL (100 mg/kg, p.o.) or vehicle (VEH; 1.25% hydroxypropyl methyl cellulose, 0.1% dioctyl sodium sulfosuccinate, and 0.25% methyl cellulose in water) at ZT18 and left undisturbed until time to perform memory tasks (see below). For the SRM task, most rats were also administered ALM, ZOL, and VEH p.o. at the concentrations above. However, one cohort of rats was administered drugs i.p. For these rats, ALM was administered at 100 mg/kg ($N = 6$), ZOL at 30 mg/kg ($N = 8$) and VEH ($N = 7$). ZOL is approximately 3X more potent i.p. than p.o.(Vanover et al., 1999) while ALM is equipotent through both routes of administration. Analysis of the sleep/wake data confirmed the equipotent effects of both drugs through both routes of administration at the concentrations tested.

WATER MAZE

All water maze (WM) tasks occurred in a pool 68″ in diameter and 25″ in depth, containing water at 24 ± 2°C made opaque by the addition of non-toxic, water soluble black paint and milk powder. Since all tests took place during the dark period, distinctive spatial cues were made of small "rice" lights colored blue, yellow, and green. Patterns of lights in distinct shapes (circle, square, diamond, "T" shape) were clearly visible from within the pool. Preliminary studies determined the minimum number of lights that were needed for learning to occur. A 10 cm diameter platform was submerged approximately 1 cm below the surface of the water in one of 6 locations (**Figure 1**). The platform location determined the orientation of the 4 quadrants used for analysis. Both WM tasks were similar to previous reports (Wenk, 2004; Ward et al., 2009).

TEST OF SPATIAL REFERENCE MEMORY

The acquisition phase occurred in one session consisting of 12–15 consecutive trials with a 60 s inter-trial interval. For each trial,

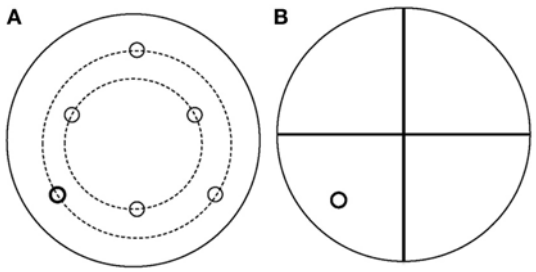

FIGURE 1 | Schematic of the water maze apparatus used for both spatial reference and spatial working memory tasks. (A) Schematic of the platform locations. **(B)** Example of quadrant orientations used for analysis used for the platform indicated in bold. Quadrant locations were always oriented so that the platform was central within a quadrant.

rats were placed in the WM facing the wall in one of three quadrants that did not contain the hidden platform. The location of the hidden platform remained constant across all trials. Rats were given 60 s to locate the platform. If the rats did not locate the platform within this period, they were guided to the platform location. When the rats reached the platform, they were allowed to remain on the platform for approximately 15 s before being placed in a dry holding cage for the next 60 s. This training sequence continued until the rats learned the task, typically 12–15 trials.

On the following day, rats were dosed with ALM, ZOL or VEH at ZT18 and a retention probe trial was performed 90 min later in which the rats were returned to the WM but the platform had been removed. A total of 40 rats were subjected to SD for 6 h prior to drug administration, and 42 were left undisturbed during this period (**Table 1**). Rats were started in the quadrant opposite the target quadrant and allowed to swim for 30 s. All trials were recorded by video camera and analyzed with Ethovision XT software (Noldus, Leesburg, VA). Test measures for the retention probe were time spent in target quadrant, latency to target quadrant, frequency of entrance into target quadrant, and total distance traveled. Swim speed was calculated to control for nonspecific effects.

TEST OF SPATIAL WORKING MEMORY

The SWM task consisted of 6 pairs of trials, one for each platform location (**Figure 1A**). In the first trial, a cued platform marked with a flag was placed in one of 6 positions in the WM. Rats were released facing the wall from one of the 3 quadrants not containing the platform and were allowed 120 s to locate the cued platform before the researcher guided the rats to the platform. This procedure provided all rats the opportunity to learn the platform location even if they did not find it on their own. After 15 s on the platform, the rats were removed from the WM and placed in a holding cage. The flag was then removed but the platform remained in the same location as in the first trial. Following a delay of 1, 5, or 10 min in the holding cage, the rats were placed back in the WM into one of the 2 quadrants that did not contain the platform and was not the starting quadrant during the first trial. Once the rats found the platform, they were removed after approximately 5 s and placed back in a holding cage for 10 min before a new pair of trials with a novel platform location was given. The order of delays was counterbalanced so that each rat was tested twice at 1, 5, or 10 min delays between the cued and hidden platforms. All trials were recorded by video camera and analyzed with Ethovision XT software (Noldus, Leesburg, VA). Test measures were time to locate the platform and the swim velocity during all tests.

STATISTICAL ANALYSIS

Statistical analyses were performed using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA). Sleep/wake data (W, NREM, and REM time) were analyzed in 30 min bins and compared between drug groups using Two-Way mixed-model ANOVA on factors "drug group" (between subjects) and "time" (within subjects). SRM performance parameters (latency, duration and frequency in target quadrant, total distance traveled) were analyzed using a One-Way ANOVA. SWM performance measures (velocity, time to platform, percent found) by delay time were analyzed using Two-Way mixed-model ANOVA on factors "drug group" (between subjects) and "time" (within subjects). Significance levels were set at $\alpha = 0.05$. When ANOVA indicated significance, Bonferroni *t*-tests were used for *post hoc* analyses.

RESULTS

Drug concentrations were chosen to be equipotent at sleep promotion based on our previous experience (Morairty et al., 2012). Although ZOL produced a more rapid onset to sleep under both SD and undisturbed conditions (No SD: ZOL = 6.6 min, VEH = 32.2 min, ALM = 25.4 min; SD: ZOL = 5.9 min, VEH = 20.0 min, ALM = 15.5 min), ALM- and ZOL-treated rats slept equivalent amounts during the last hour before the WM test (**Figure 2**; No SD: ZOL = 69.4%, ALM = 62.3%, VEH = 37.6%; SD: ZOL = 69.6%, ALM = 71.5%, VEH = 52.0%).

TEST OF SPATIAL REFERENCE MEMORY

For all performance measures analyzed, rats treated with ZOL showed significant impairments while ALM- and VEH-treated rats were indistinguishable (**Figure 3**). Following ZOL, the latency to the target zone increased (No SD: ZOL = 14.1 s, VEH = 5.7 s, ALM = 5.8 s; SD: ZOL = 18.4 s, VEH = 4.2 s, ALM = 3.6 s) and the duration in the target zone (No SD: ZOL = 5.5 s, VEH = 8.4 s, ALM = 7.9 s; SD: ZOL = 4.8 s, VEH = 7.7 s, ALM = 7.8 s), frequency entering the target zone (No SD: ZOL = 1.2, VEH = 2.7, ALM = 2.5; SD: ZOL = 0.9, VEH = 2.8, ALM = 2.9) and the distance traveled (No SD: ZOL = 472 cm, VEH = 666 cm, ALM = 725 cm; SD: ZOL = 343 cm, VEH = 709 cm, ALM = 775 cm) all decreased compared to VEH and ALM-treated rats. ALM-treated rats did not differ from VEH-treated rats on any of these four measures. Performance in the SRM task was not significantly affected by 6 h SD for any measure within any group.

Swim patterns in the WM were different for ZOL-treated rats compared to VEH- and ALM-treated rats (**Figure 4**). Both VEH and ALM rats repeatedly swam across the WM and typically swam through the area where the hidden platform was present on the previous day (**Figure 4A**). In contrast, ZOL-treated rats primarily swam around the perimeter of the WM, a pattern typical of a rat during its first exposure to the WM.

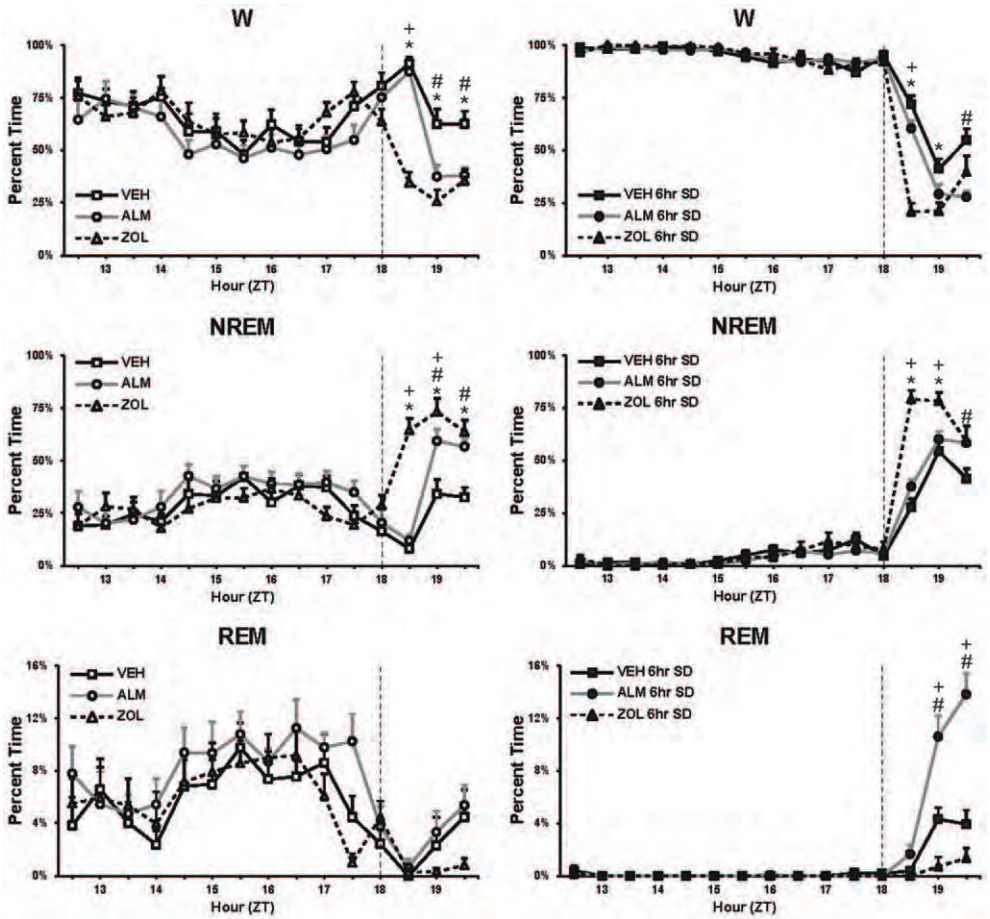

FIGURE 2 | Percent time spent in W, NREM, and REM during baseline (left panels) and during 6 h SD (right panels). The vertical line in each panel at ZT18 depicts the time of drug administration. At the end of the recording time displayed in these panels, rats were tested in the water maze. Note that, for the 60 min prior to testing (ZT19.5), the ALM and ZOL groups slept similar amounts. *, ZOL different from VEH; +, ZOL different ALM; #, ALM different from VEH; $p < 0.05$.

TEST OF SPATIAL WORKING MEMORY

ZOL-treated rats performed poorly in the SWM task compared to either VEH- or ALM-treated rats (**Figures 5**, **6**). ZOL-treated rats took longer to find the platform (No SD: ZOL = 43.4–47.3 s, VEH = 20.6–30.0 s, ALM = 22.5–30.7 s; SD: ZOL = 48.0–55.5 s, VEH = 26.9–31.0 s, ALM = 25.6–28.2 s) and swam more slowly (No SD: ZOL = 14.0–14.2 cm/s, VEH = 18.0–19.6 cm/s, ALM = 18.9–20.4 cm/s; SD: ZOL = 9.9–10.9 cm/s, VEH = 15.7–16.8 cm/s, ALM = 17.5–18.1 cm/s) than the VEH or ALM rats (**Figure 5**). These measures were not affected by increasing the delay from 1 to 5 min or 10 min for any of the 6 groups of rats.

The goal for the SWM task was to locate the platform. VEH- and ALM-treated rats found the platform the majority of the time in both SD and undisturbed conditions (83.3–100% for VEH and 79.2–87.5% for ALM; **Figure 6**). Conversely, ZOL-treated rats failed to find the platform most of the time (22.7–50.0% success rate). Interestingly, ZOL-treated rats also often failed to find the cued platform during the training phase of each pair of trials (**Figure 7**). The ZOL-treated rats in the baseline group found the cued platform 54.4% of the time while the SD ZOL-treated group were successful 53.8% of the time as compared to 98.6% for ALM-treated rats in the baseline group and 100% following SD and 100% of the time for all VEH-treated rats. A trend toward improved performance was observed with progressive trials in the ZOL-treated rats.

DISCUSSION

Though differing in the latency to induce sleep at the doses tested, ALM, and ZOL were equally effective at promoting sleep during the 90 min period prior to performance testing and both compounds significantly increased sleep compared to VEH. ALM-treated rats were indistinguishable from VEH-treated rats in their performance of both the SRM and SWM tasks. In contrast, ZOL caused significant impairments in both tasks. Specifically, in the SRM task, ZOL increased the latency to, the duration in, and the frequency of entering the target zone. In the SWM task, ZOL increased the time to find the platform, decreased the swim velocity and decreased the success rate in finding the platform. These results support the hypothesis that dual Hcrt receptor antagonism

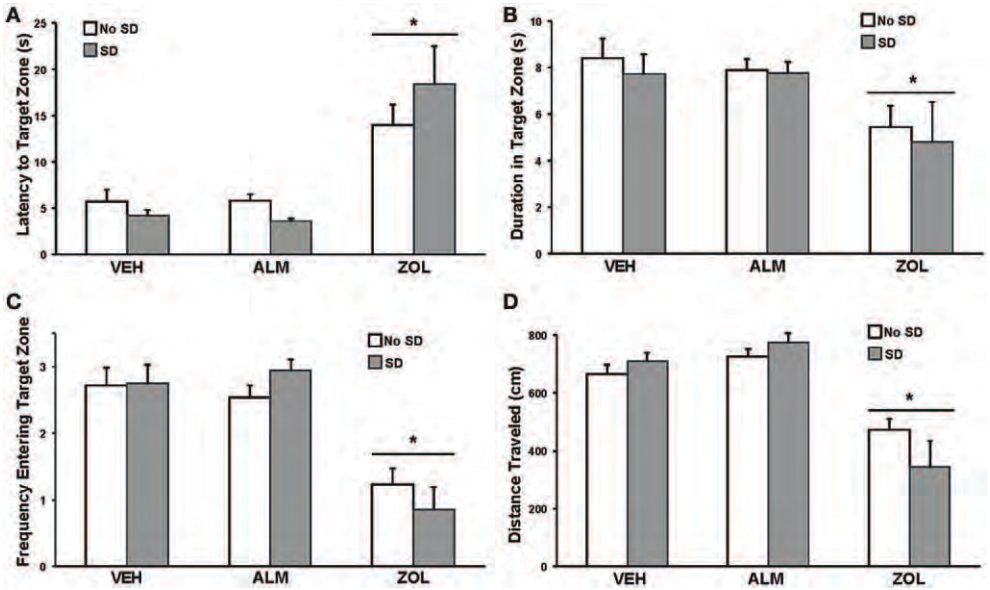

FIGURE 3 | Measures of performance in the spatial reference memory task. For all measures, ZOL-treated rats performed poorly compared to VEH- and ALM-treated rats. For all measures, the ALM-treated rats were indistinguishable from the VEH-treated rats. **(A)** Latency to the target zone. **(B)** Duration in the target zone. **(C)** Frequency entering the target zone. **(D)** Total distance traveled. For all measures, ANOVA revealed an effect of drug condition without an effect of SD. *, $p < 0.05$.

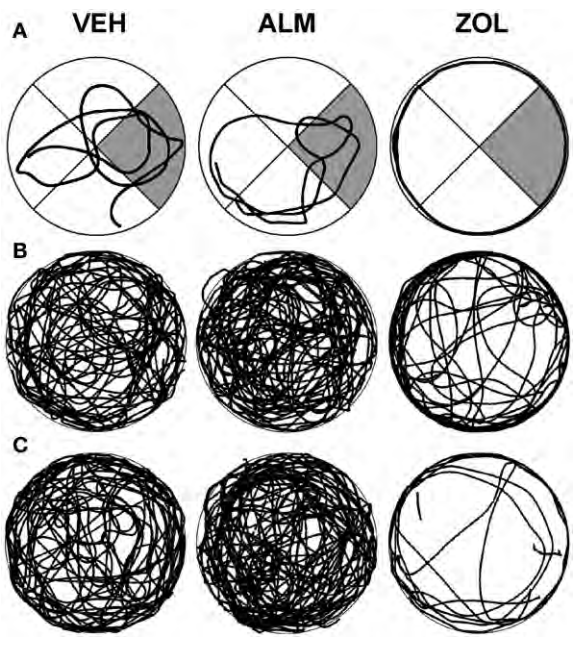

FIGURE 4 | Swim patterns during the spatial reference memory probe trials following VEH (left columns), ALM (center columns) and ZOL (right columns). (A) Examples of individual rats. The target quadrant is highlighted in gray. **(B)** Traces for all rats in the undisturbed condition. **(C)** Traces for all rats in the 6 h SD condition. Note that the searching pattern for VEH and ALM are similar while the pattern following ZOL remains primarily around the perimeter of the maze.

effectively promotes sleep without the functional impairments observed following $GABA_A$ receptor modulation.

An alternative explanation of the results obtained is that ZOL-treated rats were not motivated to perform the tasks rather than having memory/cognitive deficits. ZOL-treated rats had decreased distance traveled during the SRM task and decreased velocity during the SWM task, which could indicate a lack of motivation to escape the WM. Further, the lower success rate in finding the cued platform during the training trials for the SWM task could be interpreted as an absence of motivation to escape. However, ZOL rats did not simply float in the WM; they swam continuously, primarily circling the perimeter of the WM. As mentioned above, this swim pattern is typical of an untrained rat during its first exposure to the WM. Although not measured in this study, it is possible that the decreased distance traveled during the SRM task and decreased velocity during the SWM task are due to motor deficits produced by ZOL. This hypothesis is supported by previous studies that found prominent motor effects following ZOL administration (Depoortere et al., 1986; Steiner et al., 2011; Milic et al., 2012).

The SD protocol in these studies was included to assess whether moderate increases in sleep drive would exacerbate any cognitive deficits found following ALM or ZOL administration and also produce deficits in VEH-treated rats. While the primary active period of nocturnal rodents such as the rat is during the dark phase, rats still sleep approximately 30% of the time during this period and increasing wake duration during the dark period should create a mild sleep deficit (see **Figure 2**). Therefore, a portion of our experimental protocol involved SD during the 6 h of the dark period just prior to drug administration at ZT18.

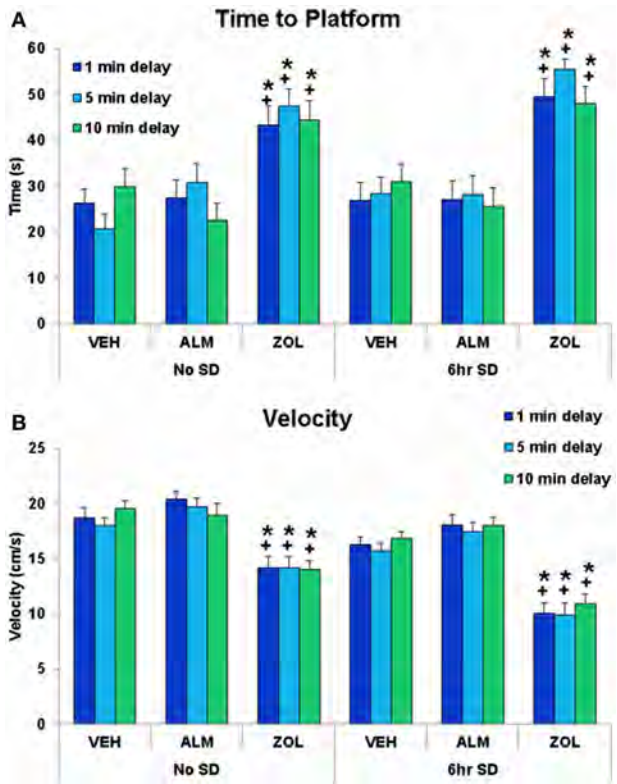

FIGURE 5 | The time to platform and the velocity swam during the spatial working memory task. (A) ZOL-treated rats found the platform significantly slower than VEH- or ALM-treated rats for all three delays following either undisturbed or SD conditions. The ALM-treated rats were not significantly different from VEH-treated rats for any condition. **(B)** ZOL-treated rats swam more slowly than either VEH- or ALM-treated rats. *, different from VEH; +, different from ALM; $p < 0.05$.

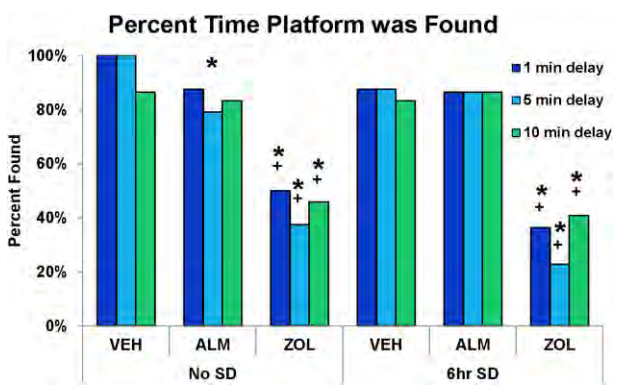

FIGURE 6 | Success rate in locating the platform during the test trials in the spatial working memory task. ZOL-treated rats found the platform significantly fewer times compared to VEH- or ALM-treated rats for all three delays and following both the undisturbed and SD conditions. In each trial, an individual rat either found or didn't find the platform; thus, there is no variation to represent as error bars in the graphs. *, different from VEH; +, different from ALM; $p < 0.05$.

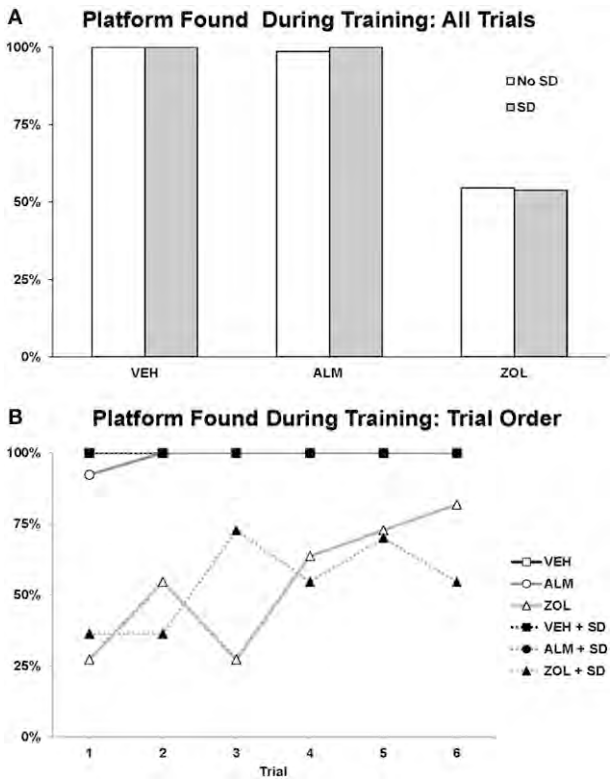

FIGURE 7 | Success rate in locating the platform during the training trials in the spatial working memory task. The platform was cued during these training trials by a flag. **(A)** The percentage of times the platform was found across all 6 training trials. **(B)** The percentage of times the platform was found trial by trial. Note that the ZOL rats tended to progressively improve across trials. In each trial, an individual rat either found or didn't find the platform; thus, there is no variation to represent as error bars in the graphs.

Although we did not find significant effects of SD vs. non-SD within any of the 3 dosing conditions, these results are likely due to the fact that we allowed the rats to sleep after drug administration until water maze testing began. This undisturbed period lasted only 60–90 min but provided an opportunity for the experimental subjects to recover from this mild sleep deprivation. If the SD were continued until testing, increased memory deficits might have been observed. Further studies are needed to determine whether this is indeed to case.

ZOL is a widely prescribed hypnotic medication that can be well-tolerated when taken as directed (Greenblatt and Roth, 2012). However, numerous adverse effects associated with ZOL usage have been reported including driving impairment (Verster et al., 2006; Gunja, 2013), effects on balance and postural tone (Zammit et al., 2008), interference with memory consolidation (Balkin et al., 1992; Wesensten et al., 1996, 2005; Mintzer and Griffiths, 1999; Morgan et al., 2010) and increased incidence of complex behaviors during sleep (Hoever et al., 2010). Some studies investigated the effects of daytime administration of ZOL and tested psychomotor function upon arousal from naps (Wesensten et al., 2005; Storm et al., 2007), a protocol which our experiments closely mimic. In these studies, ZOL or melatonin was

administered at either 10:00 or 13:00. Following a 1.5–2 h nap opportunity, subjects were awakened and required to perform a series of psychomotor and cognitive tests. Significant performance decrements were observed following ZOL in cognitive, vigilance and memory tasks while little to no decrements were found following melatonin. The results of ZOL administration on rat cognitive performance in the current study correlate well with these deficits found in humans.

In contrast, the high level of performance following ALM in both of our memory tasks suggests a high degree of safety at concentrations with hypnotic efficacy. Indeed, a recent study found no performance decrements in a variant of the WM SRM task at three-fold the concentration of ALM that we used (Dietrich and Jenck, 2010). Furthermore, another recent study found no effect of ALM at 300 mg/kg on motor function (Steiner et al., 2011). In humans, however, psychometric test battery assessment of the effect of ALM administered in the daytime found reductions in vigilance, alertness, and visuomotor and motor coordination at dose concentrations of 400–1000 mg (Hoever et al., 2010, 2012a). Notably, 400 mg ALM is within the therapeutic dose range required to improve sleep in patients with primary insomnia (Hoever et al., 2012b). Therefore, performance deficits following ALM occur within the range of hypnotic efficacy in humans. In one report, pharmacokinetic/pharmacodynamic modeling suggests that doses of 500 mg ALM and 10 mg ZOL are equivalent with respect to subjectively assessed alertness (Hoever et al., 2010). Since we find hypnotic efficacy to be achieved at roughly similar dose concentrations, there may be species differences in pharmacokinetic/pharmacodynamics of ALM and/or ZOL. While not uncommon, this makes direct translational interpretations of the present data more difficult. Regardless, in both rodents and humans, ALM appears to have a significantly better safety profile than ZOL with regards to cognitive/memory domains.

CONCLUSION

ALM and ZOL are effective hypnotics in multiple mammalian species (Brisbare-Roch et al., 2007; Hoever et al., 2010, 2012a,b; Morairty et al., 2012). They act through entirely different mechanisms of action, and their effects on cognition, psychomotor vigilance and memory are in stark contrast to one another. We found that at equipotent hypnotic concentrations, ZOL impaired SRM and SWM but ALM did not. These results support the hypothesis that antagonism of the Hcrt system can provide hypnotic efficacy without the impairments found by inducing sleep through $GABA_A$ modulation.

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Homeostatic Sleep Pressure is the Primary Factor for Activation of Cortical nNOS/NK1 Neurons

Lars Dittrich, Stephen R. Morairty, Deepti R. Warrier, Thomas S. Kilduff*

Center for Neuroscience, Biosciences Division, SRI International, Menlo Park, CA 94025

*To whom correspondence should be addressed

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Abbreviated title: Sleep pressure activates cortical nNOS neurons

ABSTRACT

Cortical interneurons, immunoreactive for neuronal nitric oxide synthase (nNOS) and the receptor NK1, express the functional activity marker Fos selectively during sleep. NREM sleep "pressure" is hypothesized to accumulate during waking and to dissipate during sleep. We reported previously that the proportion of Fos^+ cortical nNOS/NK1 neurons is correlated with established electrophysiological markers of sleep pressure. Since these markers covary with the amount of NREM sleep, it remained unclear whether cortical nNOS/NK1 neurons are activated to the same degree throughout NREM sleep or whether the extent of their activation is related to the sleep pressure that accrued during the prior waking period. To distinguish between these possibilities, we used hypnotic medications to control the amount of NREM sleep in rats while we varied prior wake duration and the resultant sleep pressure. Drug administration was preceded by 6 h of sleep deprivation ("high sleep pressure") or undisturbed conditions ("low sleep pressure"). We find that the proportion of Fos^+ cortical nNOS/NK1 neurons was minimal when sleep pressure was low, irrespective of the amount of time spent in NREM sleep. In contrast, a large proportion of cortical nNOS/NK1 neurons was Fos^+ when an equivalent amount of sleep was preceded by sleep deprivation. We conclude that, while sleep is necessary for cortical nNOS/NK1 neuron activation, the proportion of cells activated is dependent upon prior wake duration.

INTRODUCTION

Although the functions of sleep remain controversial, one of the strongest arguments for its fundamental importance is its homeostatic regulation. Sleep homeostasis refers to compensatory increases in sleep amount, sleep consolidation and/or sleep intensity that occur in response to a period of extended wakefulness (Borbely and Achermann, 2000). In mammals and birds, sleep intensity, usually assessed by measuring the spectral power of the EEG in the delta frequency range (0.5-4.5 Hz) during non-rapid eye movement sleep (NREM), is used as an index of the hypothetical sleep "pressure" that has accumulated during wakefulness (Borbely and Achermann, 2000; Rattenborg *et al*, 2009).

Despite the widespread occurrence of sleep homeostasis among animal species, our understanding of the underlying mechanisms is incomplete. Current hypotheses implicate sleep factors such as adenosine or cytokines that accumulate during waking and increase the propensity and depth of sleep (Coulon *et al*, 2012; Krueger *et al*, 2008; Landolt, 2008; Porkka-Heiskanen and Kalinchuk, 2011; Szymusiak and McGinty, 2008). Sleep factors are thought to act by inhibiting wake-promoting neurons (Porkka-Heiskanen and Kalinchuk, 2011; Rainnie *et al*, 1994), but may also act directly on the cerebral cortex (Clinton *et al*, 2011; Szymusiak, 2010). We have recently described a population of cortical GABAergic interneurons that is specifically activated during sleep (Gerashchenko *et al*, 2008; Pasumarthi *et al*, 2010). These neurons are identified by colocalized immunoreactivity for neuronal nitric oxide synthase (nNOS) and the substance P (SP) receptor NK1 (Dittrich *et al*, 2012). Activation of these neurons, assessed by immunoreactivity for the functional activity marker Fos, correlates with time spent in NREM sleep as well as with NREM delta power (Gerashchenko *et al*, 2008; Morairty *et al*, 2013). Prior wake duration "dose-dependently" increased the proportion of Fos-labeled nNOS neurons when rats were subjected to 2h, 4h, or 6h of sleep deprivation (SD) followed by a 2h recovery sleep (RS) opportunity (Morairty *et al*, 2013). Based on these observations, we have suggested that cortical nNOS/NK1 neurons are inhibited by wakefulness and activated by sleep pressure (Kilduff *et al*, 2011).

In our previous studies, we increased sleep pressure by increasing the amount of

prior wakefulness, which resulted in a compensatory increase in the amount of NREM sleep during RS. Therefore, we could not distinguish whether the time spent in NREM sleep or the magnitude of sleep pressure produced by prolonging wakefulness was the primary factor driving Fos expression in cortical nNOS/NK1 neurons. Fos expression typically reflects neuronal activity occurring during the 1-2 h prior to sacrifice (Hoffman and Lyo, 2002; Zangenehpour and Chaudhuri, 2002). If cortical nNOS/NK1 neurons are uniformly activated throughout NREM sleep and inactive during wakefulness, Fos expression in these neurons should depend on the time spent in NREM during the 1-2 h preceding sacrifice and thus would only indirectly correlate with measures of sleep pressure. Here, we sought to distinguish between these alternatives by dissociating the occurrence of NREM from the magnitude of sleep pressure using hypnotic medications to pharmacologically control NREM sleep duration under conditions in which the prior sleep/wake history was varied. To ensure that our results were not drug-specific, we utilized hypnotics with different mechanisms of action: the dual hypocretin/orexin receptor antagonist almorexant (ALM) and the $GABA_A$ receptor modulator zolpidem (ZOL). We find that, when time spent in NREM sleep is held constant, the proportion of cortical nNOS/NK1 cells activated is dependent upon prior sleep/wake history and that Fos expression in cortical nNOS/NK1 neurons reflects time kept awake (and, presumably, the accompanying sleep pressure) more robustly than any other parameter of NREM sleep.

MATERIALS AND METHODS

Animals

A total of 39 male Sprague-Dawley rats were studied. Animals were housed in separate cages in temperature-controlled recording chambers (20-24^{o}C, 30-70% relative humidity) under a 12/12 light/dark cycle with food and water available *ad libitum*. The weights at experiment were 587g ± 63 (mean ± SD). All experimental procedures involving animals were approved by SRI International's Institutional Animal Care and Use Committee and were in accordance with National Institute of Health (NIH) guidelines.

Surgical procedures

Surgical procedures involved implantation of sterile telemetry transmitters (F40-EET, Data Sciences Inc., St Paul, MN) as previously described (Morairty *et al*, 2013; Morairty *et al*, 2008; Morairty *et al*, 2012). Briefly, transmitters were placed intraperitoneally under isoflurane anesthesia. Biopotential leads were routed subcutaneously to the head and neck. EEG electrodes were placed epidurally 1.5 mm anterior to bregma and 1.5 mm lateral to midline, and 6 mm posterior to bregma and 4 mm lateral to midline on the right hemisphere. EMG leads were positioned bilaterally through the nuchal muscles.

Identification of Sleep/Wake States and Sleep/Wake Data Analyses

Behavioral state determinations and data analyses were conducted as previously described (Morairty *et al*, 2013; Morairty *et al*, 2012). After at least 3 weeks post-surgical recovery, EEG and EMG were recorded via telemetry using DQ ART 4.1 software (Data Sciences Inc., St Paul, MN). Following completion of data collection, the EEG and EMG recordings were scored in 10 s epochs as waking, rapid eye movement sleep (REM), or non-rapid eye movement sleep (NREM) by expert scorers who examined the recordings visually using NeuroScore software (Data Sciences Inc., St Paul, MN). For calculation of bout durations, a bout was defined as consisting of a minimum of two consecutive epochs of a given state and ended with any single state change epoch. EEG spectra were analyzed with a fast Fourier transform algorithm using a Hanning Window without overlap (NeuroScore software, Data Sciences Inc., St Paul, MN) on all epochs without artifact. For comparisons of EEG spectra, average spectra of a specific state were normalized to the average spectra of the respective state during a 6 h baseline recording (Zeitgeber time 0-6, or ZT0-ZT6). For calculation of NREM EEG delta power (NRD), the mean of the power between 0.5–4.5 Hz of the averaged NREM spectra was calculated and normalized to the respective value of the 6 h baseline recording. NRD energy (NRDE) was calculated by multiplying the time (h) spent in NREM sleep by the normalized NRD power.

Detection of individual slow waves was adapted from (Vyazovskiy *et al*, 2007). Raw EEG was bandpass filtered (0.5-4.5 Hz) using the bandpassfilter.m function from

the FieldTrip toolbox (http://www.ru.nl/neuroimaging/fieldtrip) in MATLAB (Mathworks, Natick, MA). The first positive peak after a zero crossing was identified as a single slow wave. The slope was approximated as a straight line between that peak and the last negative peak preceding the zero crossing. All slopes from artifact-free NREM epochs were averaged for each rat. Slopes were normalized to the average NREM slopes from the respective baseline recordings.

Sleep deprivation procedures

Animals were continuously observed while EEG and EMG were recorded and, when inactive and appeared to be entering sleep, cage tapping occurred. When necessary, an artist's brush was used to stroke the fur or vibrissae. After ZOL, it was sometimes necessary to touch rats to keep them awake.

Experimental Protocol

The rats were assigned to six groups: (1) VEH with low sleep pressure (*n*=6); (2) VEH with high sleep pressure (*n*=7); (3) ZOL with low sleep pressure (*n*=6); (4) ZOL with high sleep pressure (*n*=7); (5) ALM with low sleep pressure (*n*=6); and (6) ALM with high sleep pressure (*n*=7). Dosing occurred at ZT12, 100 mg/kg p.o. in 2 ml/kg for both drugs. Perfusion occurred at ZT14 for VEH and ZOL groups and at ZT14.5 for ALM groups due to its longer latency to sleep onset (Black *et al*, 2013; Morairty *et al*, 2012; Morairty *et al*, 2014). Rats in the high sleep pressure conditions were sleep deprived during the 6 h prior to dosing (Figure 1).

Immunohistochemical procedures

Rats were killed with an overdose of euthanasia solution i.p. (SomnaSol, Butler-Schein, Dublin, OH) and transcardially perfused with heparinized phosphate buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were postfixed overnight in the same fixative and then immersed in 30% sucrose in phosphate buffered saline until they sank. Coronal brain sections were cut at 40 µm thickness. Double immunohistochemistry for Fos and nNOS was performed on serial sections of rat brain as described previously (Gerashchenko *et al*, 2008; Pasumarthi *et al*, 2010). Sections

were first incubated overnight with rabbit anti-Fos antibody (1:4000-5000, sc-52, Santa Cruz Biotechnology, Dallas, TX), then with biotinylated donkey anti-rabbit antibody (1:500, Jackson ImmunoResearch, West Grove, PA), followed by avidin-biotinylated horseradish peroxidase complex (1:200, PK-6100, Vector Laboratories, Burlingame, CA), and nickel-enhanced 3,3' diaminobenzidine (SK4100, Vector Laboratories) for a black reaction product. nNOS neurons were stained in the same sections by overnight incubation in rabbit-nNOS antibody (1:2000, 61-7000, Invitrogen, Camarillo, CA), followed by biotinylated donkey anti-rabbit antibody and avidin-biotinylated horseradish peroxidase complex, and visualized with NovaRED (SK-4805, Vector Laboratories) for a red-brown reaction product.

Cell Counting

Single-labeled nNOS and double-labeled Fos^{+}/nNOS cells were counted in one hemisection each at 1.4 mm anterior, 0.5 mm posterior, and 3.0 mm posterior to bregma (Paxinos *et al*, 1999). The percentage of nNOS neurons expressing Fos was calculated as described previously (Gerashchenko *et al*, 2008; Pasumarthi *et al*, 2010). Micrographs for publication were taken at 200x magnification on a Leica DM 5000B microscope (Leica Microsystems, IL) with a Microfire S99808 camera (Optronics, CA) in Stereoinvestigator (MBF Bioscience, Williston, VT). Adjustments of brightness, color or contrast were applied to the whole image and performed in Photoshop (Adobe Systems, San Jose, CA).

Statistics

Statistical tests were performed using Excel (Microsoft, Redmond, WA), MATLAB and R (R Foundation for Statistical Computing). For each studied variable, we used Mann-Whitney U-tests to test whether it significantly distinguished between the high sleep pressure and low sleep pressure groups within the same drug treatment condition. The NREM bout duration histograms (Figure 2) were tested with 2-way permutation ANOVA (Manly, 2007) with 5000 iterations of the factors "bout duration" and "sleep pressure". If an interaction was found, the sleep pressure conditions were compared for each bout duration using Holm-Sidak corrected t-tests. For comparison of EEG power spectra, we

first performed 2-way permutation ANOVA with 5000 iterations with factors “frequency bin” and “sleep pressure”. If interactions were found, the source of the interaction was evaluated through bin-by-bin (0.122 Hz) uncorrected t-tests between the sleep pressure conditions. Only coherent changes that affected a range of frequencies were considered potentially meaningful, whereas isolated bins with significant changes were ignored. To determine how strongly different physiological parameters distinguished the high vs. low sleep pressure groups, we calculated Hedges’ g (difference of the means divided by pooled standard deviation) and the 95% confidence intervals as an effect size measure (Hedges and Olkin, 1985). Since Hedges’ g is a parametric measure, data were first transformed to achieve a normal distribution. The percent time in NREM and the %Fos^{+}/nNOS neurons were arcsine transformed. NREM bout durations, NRD, slow wave slopes (each normalized by respective baseline values), and NRDE were $\log_{10}$ transformed. Normal distributions after data transformation were verified visually using normal probability plots. To test whether Hedges’ g for %Fos/nNOS was significantly different from Hedges’ g for any other variable, g was first transferred to Fisher’s z (Borenstein *et al*, 2009). The z for %Fos/nNOS was then compared to z for every other variable (Meng *et al*, 1992) and the p-values were Holm-Sidak-corrected for multiple comparisons.

RESULTS

Sleep time can be dissociated from sleep/wake history using hypnotics

After experimental manipulation of sleep pressure as illustrated in Figure 1, the VEH-dosed rats showed the expected influence of waking history on sleep propensity: whereas undisturbed rats showed an increased time spent awake beginning at lights off (ZT12), rats that were sleep deprived during the preceding 6 h showed strongly reduced wakefulness at the same time of day (Figure 2A). ALM decreased the time spent awake in both groups but, at the dose used, the sleep deprived rats showed a stronger reduction of wakefulness than rats that were undisturbed for the 6 h preceding dosing, indicating an additive effect of ALM and sleep pressure (Figure 2B). In contrast, ZOL

caused a strong reduction of wakefulness irrespective of the preceding wake history (Figure 2C). Since we previously found that Fos expression in rat cortical nNOS neurons is dependent on NREM time during the 90 min preceding transcardial perfusion (Morairty *et al*, 2013), we focused on that time window for the following analyses. Figure 2D-F depicts the time each rat spent in wake, NREM, and REM during the 90 min immediately preceding sacrifice. Whereas the time spent in wake, NREM and REM differed between the low and high sleep pressure groups treated with either VEH or ALM, these physiological parameters did not differ between the groups treated with ZOL indicating a decoupling between prior sleep/wake history and vigilance states with ZOL treatment.

ZOL disrupts established measures of sleep pressure

Given the results in Figure 2D-F, we evaluated whether the high and low sleep pressure groups could be distinguished after ALM and ZOL treatment using four established measures of sleep pressure: NREM bout duration, NREM delta power (NRD), NREM delta energy (NRDE), and slow wave slopes. NREM bout duration frequency histograms were shifted towards longer bout durations in the sleep deprived groups (Figure 2G-I). Although there was a significant interaction between the factors "bout duration" and "sleep pressure" following VEH ($F_{5,55}$=6.74, p=0.007) and ALM ($F_{5,55}$=4.60, p=0.0008), this interaction did not reach statistical significance for ZOL ($F_{5,55}$=2.28, p=0.056). Accordingly, the average NREM bout durations were longer for sleep deprived than undisturbed rats following VEH (p=0.002) and ALM (p=0.008), but not for ZOL (p=0.073; Figure 2J).

As expected, the NREM EEG power spectra showed an elevated power in the delta range in the high sleep pressure group for VEH-dosed rats (Figure 3B). The wake and NREM spectra for ALM-dosed rats resembled those of VEH-dosed rats, including the increased NREM delta power in the high sleep pressure group (Figure 3C, D). Following ZOL, wake and NREM spectra were strongly altered compared to the corresponding baseline recordings, as indicated by the deviations from the basal value 1 in Figure 3E, F. In contrast to VEH and ALM, neither a main effect of "sleep pressure" nor an interaction of "frequency bin" and "sleep pressure" was found following ZOL for

either wake or NREM spectra, indicating that spectral power did not depend on prior sleep/wake history. (REM spectra are not shown because, in some groups, too little REM occurred to calculate representative spectra). Figure 3G, H depicts normalized NRD (0.5-4.5 Hz) and NRDE for each rat. Both measures significantly distinguished the high vs. low sleep pressure groups following VEH ($p=0.005$ for NRD, $p=0.005$ for NRDE) as well as ALM ($p=0.022$ for NRD, $p=0.001$ for NRDE) treatment, whereas no difference was found following ZOL ($p=1$ for NRD, $p=0.63$ for NRDE).

Lastly, we measured the average slopes of individual EEG slow waves during NREM. As expected, the slow wave slopes were steeper for sleep deprived rats than for undisturbed rats following VEH (Figure 4B). This difference was preserved after ALM (Figure 4C) but not after ZOL (Figure 4D). Consequently, the average slow wave slope was significantly greater in the high sleep pressure than in the low sleep pressure group following VEH ($p=0.008$) and ALM ($p=0.001$) but not following ZOL ($p=0.366$; Figure 4E).

Fos expression in nNOS neurons depends on prior sleep/wake history and resultant sleep pressure

To determine whether the percentage of Fos^+ cortical nNOS neurons depends on prior sleep/wake history or only on NREM time during the 90 min preceding sacrifice, we performed double-immunohistochemistry for Fos and nNOS. As depicted in Figure 5A-E, sleep deprived rats showed higher levels of %Fos/nNOS than undisturbed rats irrespective of drug treatment. Consequently, %Fos/nNOS significantly distinguished between the high vs. low sleep pressure conditions following VEH ($p=0.001$), ZOL ($p=0.001$) and ALM ($p=0.001$; Figure 5E). Notably, the separation between conditions was absolute after each drug treatment, i.e., there were no overlapping data points.

Fos/nNOS is the best indicator of sleep/wake history and resultant sleep pressure

To determine which physiological measure was most closely related to prior sleep/wake history, we quantified the effect sizes (Hedges' g) for the difference between the high vs. low sleep pressure groups for each of the parameters measured in the present study

(see Methods). For each drug treatment, Hedges' g was greatest for %Fos/nNOS (Figure 5F-H). Following VEH, %Fos/nNOS separated the sleep pressure groups significantly better than bout duration, NRD, or NRDE (Figure 5F) and better than all parameters following ALM (Figure 5G).

DISCUSSION

These results demonstrate that the extent of activation of cortical nNOS/NK1 neurons is determined by prior sleep history. When sleep pressure is assumed to be low, cortical nNOS neurons are largely inactive (Figure 5A-E) even in the presence of high amounts of NREM sleep as illustrated by the ZOL group in Figure 2E.

%Fos/nNOS depends on sleep/wake history

Using hypnotic treatment, we were able to dissociate time spent asleep during the 90 min before sacrifice from the prior sleep/wake history. Following ZOL, neither time spent in wake, NREM, nor REM differed between rats that were sleep deprived and rats that were left undisturbed for the preceding 6 h. Nonetheless, in ZOL-treated rats, the proportion of Fos^+ cortical nNOS neurons was significantly greater in the high sleep pressure than the low sleep pressure group. We conclude that cortical nNOS neurons are not activated simply by the occurrence of NREM sleep, rather, the %Fos/nNOS depends on the magnitude of sleep pressure that has accumulated during the time preceding sleep onset. Together with our previous studies (Gerashchenko *et al*, 2008; Morairty *et al*, 2013), these results demonstrate that cortical nNOS neurons are responsive to homeostatic sleep drive.

We found that the proportion of Fos^+ cortical nNOS neurons was a better indicator of prior sleep/wake history than total time spent in NREM, average NREM bout duration, NREM delta power, or the average slope of NREM slow waves (Figure 5F-H). Following ZOL, %Fos/nNOS was the only measure that significantly distinguished between the low and high sleep pressure groups. This observation makes it unlikely that Fos expression in cortical nNOS neurons is downstream of any of these variables

(e.g., driven by slow wave activity), although experimental confirmation will depend on the ability to selectively manipulate cortical nNOS/NK1 neurons.

In agreement with our previous findings (Morairty *et al*, 2014), the hypnotic efficacy of ALM and ZOL was comparable at the doses used in the present study. Nonetheless, the same dose of ALM produced different amounts of sleep in the high sleep pressure and low sleep pressure groups. This result is consistent with the view that ALM removes a wake-inducing input – Hcrt tone – whereas ZOL actively inhibits neuronal activation. Rather unspecific neuronal inhibition might bias the system towards sleep, whereas Hcrt antagonism might just impair the ability to stay awake in the presence of endogenous sleep pressure. A more detailed comparison will be needed to test if this is indeed a qualitative difference between the drugs or an effect of non-equivalent doses. Nonetheless, despite increased NREM sleep, ALM did not increase levels of Fos in cortical nNOS neurons in the low sleep pressure condition, which is consistent with the results obtained from the ZOL experiment.

nNOS/NK1 neurons and NREM delta power

The finding that, following ZOL, NRD did not differ between sleep deprived and undisturbed rats despite the pronounced difference in %Fos/nNOS between groups was surprising, since we have previously found that activation of these neurons may facilitate NRD (Morairty *et al*, 2013). Therefore, we had expected that increased Fos expression in cortical nNOS neurons would coincide with increased NRD in conditions when total NREM time did not differ. Given the pronounced effects of ZOL on the EEG, it is conceivable that ZOL masked the effects of cortical nNOS neuron activation on the NREM EEG. The low frequencies of the NREM power spectra were conspicuously increased following ZOL irrespective of sleep pressure (Figure 3D). Since cortical nNOS neurons are GABAergic (Kubota *et al*, 2011), the $GABA_A$ modulator ZOL might act directly on the downstream targets of these neurons. This interpretation is in agreement with the finding that the sleep pressure-dependent modulation of the NREM EEG was not impaired by the Hcrt antagonist ALM, since sleep deprived rats showed significantly elevated NRD. Based on Hedges' g, the difference in NRD between high

and low sleep pressure conditions was not smaller following ALM than VEH treatment (Figure 5).

Although the downstream targets of sleep-active cortical nNOS neurons have not yet been identified, these neurons are present in all cortical areas (Vincent and Kimura, 1992), form long-range cortico-cortical projections (Tomioka *et al*, 2005), and appear to be the origin of a dense nNOS-positive fiber network (Vincent and Kimura, 1992; Yousef *et al*, 2004) that is suited for producing a near simultaneous NO signal throughout a large cortical volume (Philippides *et al*, 2005). Thus, it seems likely that cortical nNOS neurons may exert a widespread effect on the cortex during sleep. This inference is supported by our recent finding that nNOS KO mice show deficits in regulation of delta power and consolidation of NREM sleep (Morairty *et al*, 2013). However, direct and specific experimental manipulation of these neurons will be necessary to determine the specific effects on cortical activity patterns.

Regulation of nNOS/NK1 neurons

While NREM sleep appears to be a permissive state for activation of cortical nNOS neurons, we demonstrated here that the magnitude of activation of these cells during NREM depends on prior sleep/wake history. The mechanism by which prior wake time is linked to activation of cortical nNOS neurons is of great interest, since it could provide insight into how the accumulation of sleep pressure is tracked by the brain. A better understanding of this mechanism could prove relevant for facilitating restorative sleep or combating pathological sleepiness. The integration of time spent awake might occur at the level of the nNOS/NK1 neurons themselves. Locally accumulating sleep factors, such as adenosine and cytokines, might activate these neurons (Kilduff *et al*, 2011). Another such factor could be Substance P (SP). mRNA levels of the gene coding for SP are increased in the cortex by sleep deprivation (Martinowich *et al*, 2011). Cortical nNOS neurons co-express the SP receptor NK1 and are strongly and directly activated *in vitro* by SP (Dittrich *et al*, 2012).

Fos expression in cortical nNOS/NK1 neurons remains minimal as long as rats are kept awake, irrespective of accrued sleep pressure (Morairty *et al*, 2013). Therefore, if

integration of sleep pressure indeed occurs at the level of cortical nNOS neurons, a wake-related inhibitory input must be assumed that prevents activation of these cells before sleep onset. This view is congruent with the model we have presented previously (Kilduff *et al*, 2011).

Alternatively, the integration of sleep pressure might occur upstream of cortical nNOS neurons. In this scenario, cortical nNOS neurons would receive activating input only during NREM, the magnitude of which depending on the sleep/wake history. In order to identify the mechanisms by which sleep pressure is linked to activation of cortical nNOS neurons, it will be critical to characterize the anatomical and neurochemical inputs to these cells.

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FIGURE LEGENDS

Figure 1. Experimental design. Sleep pressure increases during the active phase and decreases during the inactive phase. Rats were dosed at ZT12 (first vertical dashed line), when sleep pressure is lowest. We assume that sleep pressure increases slowly, if at all, during hypnotic-induced sleep; therefore, the corresponding curve remains low after dosing. To increase sleep pressure but keep the circadian conditions identical, rats in a second group were subjected to 6 h of sleep deprivation starting at ZT6 and dosed at ZT12. Rats in both conditions were perfused 2 or 2.5h after dosing (second vertical dashed line). Black and white bars indicate light conditions.

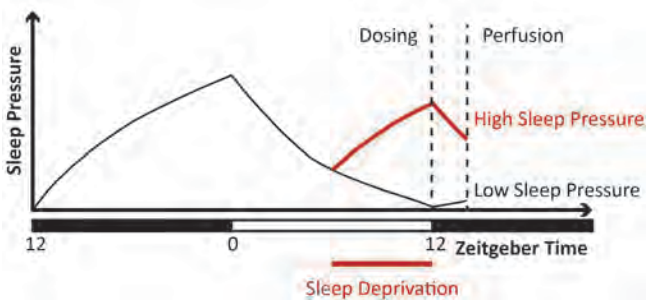
Figure 2. Time spent awake and asleep before sacrifice. **A-C**. Time spent awake between ZT6 and the time of transcardial perfusion. High sleep pressure groups were sleep deprived from ZT6-ZT12, low sleep pressure groups were left undisturbed so that the amount of sleep pressure differed. Lighting conditions are indicated below the panels. Dosing occurred at ZT12. **D-F**. Time spent in wake, NREM, and REM during the 90 min preceding sacrifice. After ZOL dosing, the time spent in any state did not differ between the sleep pressure conditions. Horizontal lines indicate group medians. *p<0.05, U-test. **G-I**. NREM bout duration frequency histograms during the 90 min preceding sacrifice following VEH (G), ALM (H) and ZOL (I) dosing. *p<0.05, Holm-Sidak test after significant interaction in permutation ANOVA. **J**. Average NREM bout durations. *p<0.05, U-test

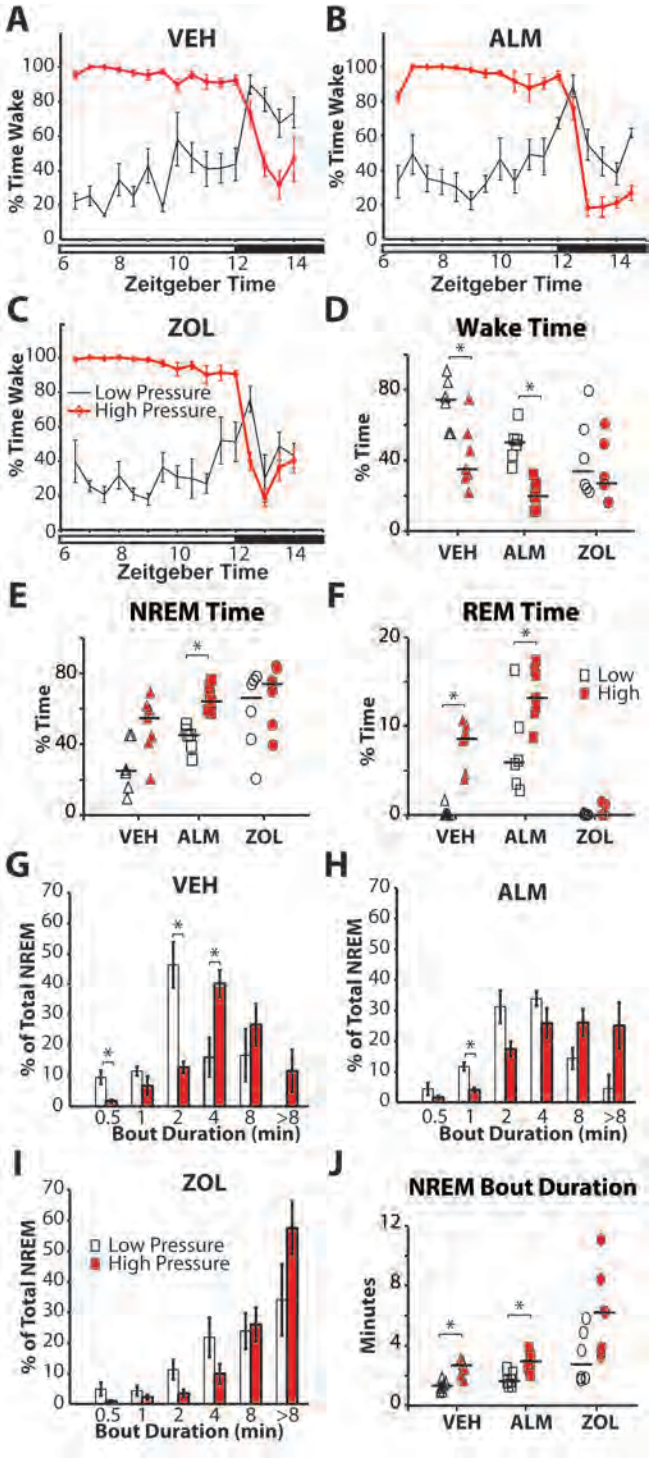
Figure 3. Spectral analyses. Wake (**A, C, E**) and NREM (**B, D, F**) EEG power spectra for the 90 min preceding sacrifice were normalized by the respective baselines (ZT0-6). Interactions of factors "frequency" and "sleep pressure" (permutation ANOVA) are indicated for each panel. The degrees of freedom are 491 and 5401 for all interactions. The p-values for *post hoc* uncorrected bin-by-bin t-tests are indicated below the spectra. Following VEH and ALM dosing, increased sleep pressure coincided with increased NREM delta power (arrows) whereas, after ZOL dosing, NREM delta power was increased irrespective of sleep pressure. **G**. Average NREM delta power (NRD) during the 90 min preceding sacrifice. **H**. NREM delta energy (NRDE) during 90 min preceding sacrifice. Both NRD and NRDE distinguished between the high and low sleep pressure groups following VEH and ALM but not following ZOL dosing. Horizontal lines indicate group medians. *p<0.05, U-test

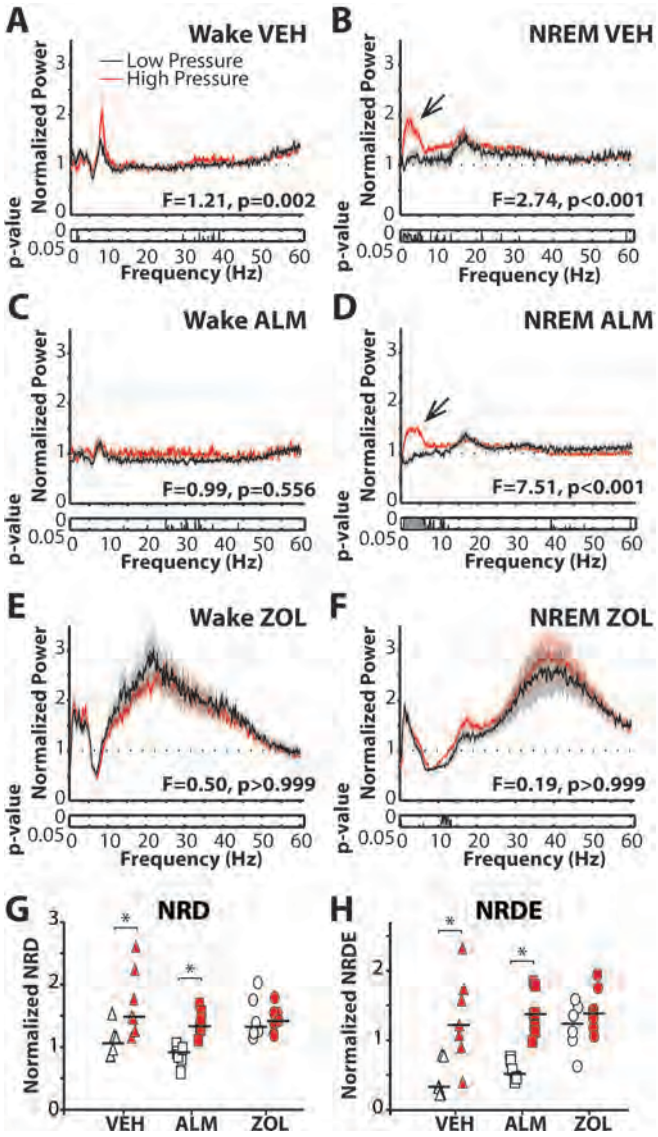
Figure 4. Slopes of NREM EEG slow waves during the 90 min preceding sacrifice. **A**. The raw EEG trace (top) was bandpass filtered in the slow wave range 0.5-4.5 Hz (bottom). Positive (green) and negative peaks (red) were identified. A straight line between a negative and a positive peak encompassing a zero crossing was defined as the slope of the respective slow wave. **B, C, D**. Average slow waves ± SEM for the experimental groups. **E**. Average NREM slow wave slopes. The slopes distinguished between the low and high sleep pressure groups following VEH and ALM but not following ZOL dosing. Horizontal lines indicate group medians. *p<0.05, U-test

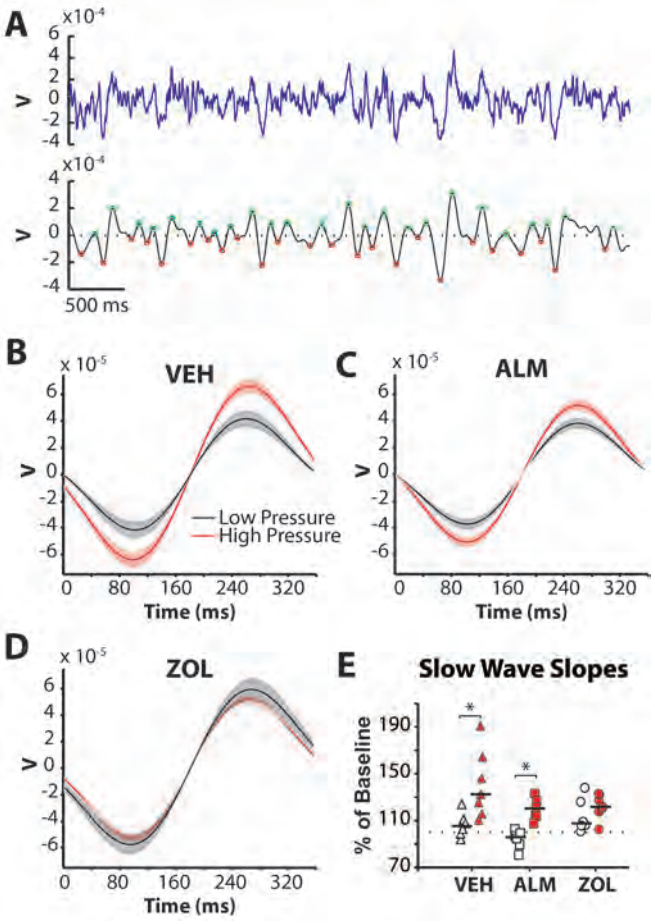
Figure 5. Fos expression in cortical nNOS neurons depends on sleep pressure. **A-D**. Example micrographs of Fos/nNOS double immunohistochemistry. Following both hypnotics, nNOS neurons were single-labeled (arrows) in low sleep pressure conditions (A, C). In the high sleep pressure conditions (B, D), many nNOS neurons were double-labeled for Fos (black triangles) irrespective of the drug treatment. Scale bar indicates 50 µm. **E**. Proportion of Fos^+ cortical nNOS neurons. Note that %Fos/nNOS

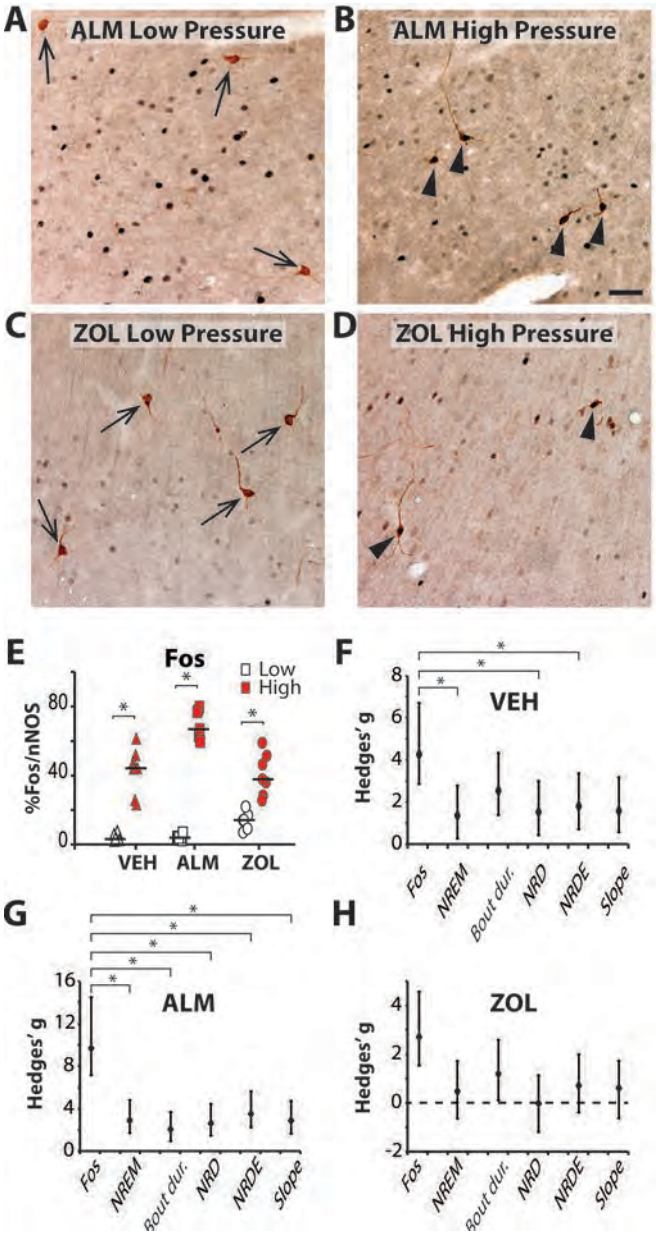
completely separated all high sleep pressure groups from the respective low sleep pressure groups. Horizontal lines indicate group medians. *p<0.05, U-test. **F-H**. Effect sizes for the difference between low and high sleep pressure groups using six different measures. Data for each of the six parameters listed on the abscissa were transformed to obtain normal distributions. For each variable, Hedges' g ± 95% confidence interval was calculated as an effect size for the difference between the two groups following VEH (F), ALM (G), and ZOL (H) treatment. Following each drug treatment, the strongest effect was seen for %Fos/nNOS. ***** indicates significantly ($p<0.05$) smaller g than that for %Fos/nNOS after Holm-Sidak correction for multiple comparisons.


Dosing
Perfusion
Sleep Pressure
High Sleep Pressure
Low Sleep Pressure
12
0
12
Zeitgeber Time
Sleep Deprivation


A
VEH
% Time Wake
Zeitgeber Time
B
ALM
% Time Wake
Zeitgeber Time
C
ZOL
Low Pressure
High Pressure
% Time Wake
Zeitgeber Time
D
Wake Time
% Time
VEH
ALM
ZOL
E
NREM Time
% Time
VEH
ALM
ZOL
F
REM Time
Low
High
% Time
VEH
ALM
ZOL
G
VEH
% of Total NREM
Bout Duration (min)
H
ALM
% of Total NREM
Bout Duration (min)
I
ZOL
Low Pressure
High Pressure
% of Total NREM
Bout Duration (min)
J
NREM Bout Duration
Minutes
VEH
ALM
ZOL


A
Wake VEH
Low Pressure
High Pressure
Normalized Power
F=1.21, p=0.002
p-value
Frequency (Hz)
B
NREM VEH
F=2.74, p<0.001
C
Wake ALM
F=0.99, p=0.556
D
NREM ALM
F=7.51, p<0.001
E
Wake ZOL
F=0.50, p>0.999
F
NREM ZOL
F=0.19, p>0.999
G
NRD
Normalized NRD
VEH
ALM
ZOL
H
NRDE
Normalized NRDE


A
x10-4
V
500 ms
B
VEH
x 10-5
Low Pressure
High Pressure
Time (ms)
C
ALM
D
ZOL
E
Slow Wave Slopes
% of Baseline
VEH
ALM
ZOL


A
ALM Low Pressure
B
ALM High Pressure
C
ZOL Low Pressure
D
ZOL High Pressure
E
Fos
Low
High
%Fos/nNOS
80
40
0
VEH
ALM
ZOL
F
VEH
Hedges' g
8
6
4
2
0
Fos
NREM
Bout dur.
NRD
NRDE
Slope
G
ALM
Hedges' g
16
12
8
4
0
Fos
NREM
Bout dur.
NRD
NRDE
Slope
H
ZOL
Hedges' g
4
2
0
-2
Fos
NREM
Bout dur.
NRD
NRDE
Slope

Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance

Proposal Log Number DR080789, Award Number W81XWH-09-2-0080

PI: Thomas Neylan, MD **Org:** NCIRE

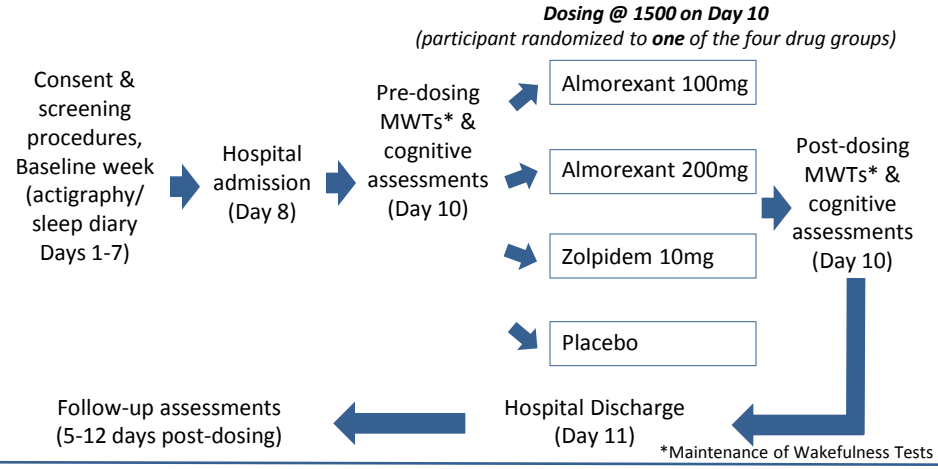
Study Objectives

To compare neurocognitive performance at peak concentration at the midpoint of the habitual wake period in subjects randomized to almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo.

Rationale/Military Relevance

In recent years, there has been increased focus on neurocognitive effects of hypnotic medications that adversely affect behavior during unanticipated awakenings during the night, especially within military personnel. Almorexant is a hypocretin/orexin antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with almorexant are easily aroused from sleep and behave free of ataxia and other impairment. If this observation is confirmed in humans, it will have substantial implications for the management of disturbed sleep in both military and civilian populations.

Study Design



Timeline and Total Cost

Subtasks	Y1	Y2	Y3	Y4	Y5	Y6
Write protocol						
Obtain scientific and human use approvals						
Purchase study related equipment/supplies						
Train laboratory personnel						
Collect data on 200 volunteers						
Score and analyze data						
Write/public final report						
Estimated Budget (Directs, $K)	345 K	645 K	672 K	618 K	689 K	452 K

Milestones

Year 1
- Protocol finalized and study documentation submitted to IRBs
- IND filed with the FDA
- Study personnel hired and trained on study protocol and procedures
- Key study equipment purchased and tested

Year 2
- Human subjects approvals obtained from UCSF CHR, VA R&D Committee, and USAMRMC HRPO
- Study drug provided by Actelion Pharmaceuticals
- 19 eligible participants identified through recruitment and screening efforts

Year 3
- New study personnel (Study Coordinator, Recruiter, and Research Assistant) hired and trained on the study protocol and procedures
- 47 eligible participants identified through recruitment and screening efforts
- Recruitment materials revised to increase phone screening success and boost enrollment

Year 4
- New study personnel (Study Coordinator, Recruiter, and Research Assistant) hired and trained on the study protocol and procedures
- At the end of Y4, 118 participants have been dosed in total; screening and enrollment ongoing

Year 5

Enrollment completed with a total of 203 participants dosed.

Year 6
- In our final year with a no-cost extension, we will enter, clean, score and analyze data and write the final public report.